AD-A166 564 AFRRI (ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE) 1/1
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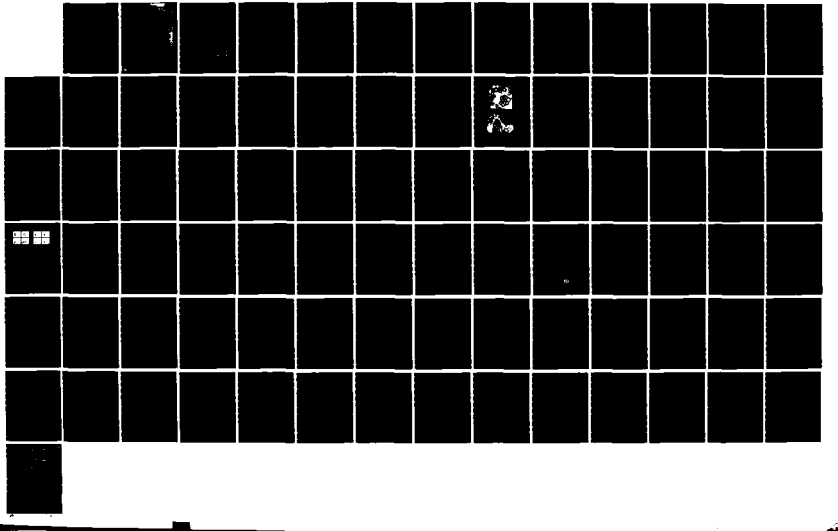

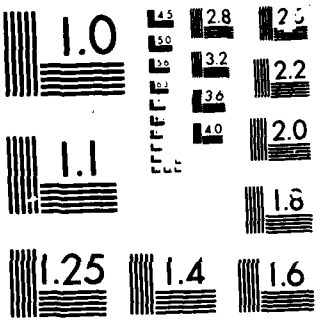

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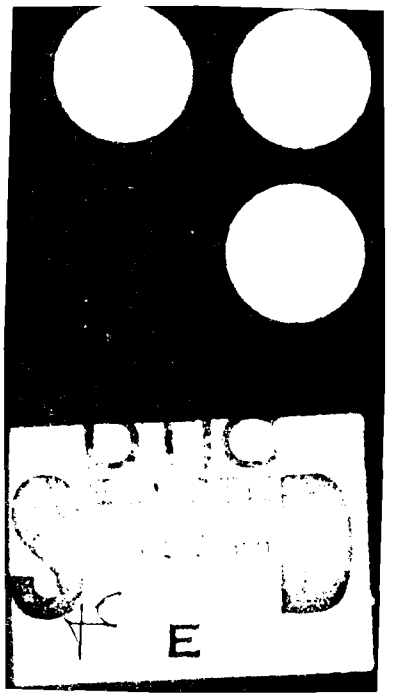
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REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): AFRRI SR85-33 through 85-41	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Armed Forces Radiobiology Research Institute; 6b. OFFICE SYMBOL (If applicable): AFRRI	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State and ZIP Code): Defense Nuclear Agency, Bethesda, Maryland 20814-5145	7b. ADDRESS (City, State and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Defense Nuclear Agency; 8b. OFFICE SYMBOL (If applicable): DNA	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State and ZIP Code): Washington, DC 20305	10. SOURCE OF FUNDING NOS. — PROGRAM ELEMENT NO.: NWED QAXM; PROJECT NO.; TASK NO.; WORK UNIT NO
11. TITLE (Include Security Classification): AFRRI Reports, Oct-Dec 1985	
12. PERSONAL AUTHOR(S)	
13a. TYPE OF REPORT: Reprints/Technical; 13b. TIME COVERED: FROM ____ TO ____	14. DATE OF REPORT (Yr., Mo., Day); 15. PAGE COUNT: 83
16. SUPPLEMENTARY NOTATION	
17. COSATI CODES — FIELD; GROUP; SUB GR.	18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): N/A

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This volume contains AFRRI Scientific Reports SR85-33 through 85-41 for Oct-Dec 1985.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS ☐	21. ABSTRACT SECURITY CLASSIFICATION: UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL: Junith A. Van Deusen	22b. TELEPHONE NUMBER (Include Area Code): (202)295-3536; 22c. OFFICE SYMBOL: ADMG

DD FORM 1473, 83 APR — EDITION OF 1 JAN 73 IS OBSOLETE

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Scientific Reports

RADIATION RESEARCH 104, 182–190 (1985)

Behavioral Toxicity and Efficacy of WR-2721 as a Radioprotectant

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BOGO, V., JACOBS, A. J., AND WEISS, J. F. Behavioral Toxicity and Efficacy of WR-2721 as a Radioprotectant, *Radiat. Res.* 104, 182–190 (1985).

S-2-(3-Aminopropylamino)ethylphosphorothioic acid (WR-2721) is a promising protectant for radiation-induced lethality. However, treatment with WR-2721 also produces nausea, vomiting, diarrhea, and hypotension, which implies severe functional consequences. Three studies were conducted to assess the effects of WR-2721 on rat motor performance and weight and to assess the ability of WR-2721 to mitigate the early performance decrement (PD) produced by ionizing radiation. In the first study, rats trained on the accelerod motor performance task were give 200, 300, or 400 mg/kg WR-2721 intraperitoneally (ip). The highest dose used referenced the maximum tolerated dose in the rat, which is two-thirds the median lethal dose (590 mg/kg). The subjects were tested immediately after treatment, at 30-min intervals for 3 h, and again at 24 h. All groups ($N = 6$/group) demonstrated a significant decrease in accelerod performance compared to control levels across the eight test trials, which ranged from 24 to 44% in the 200 and 400 mg/kg dose groups, respectively. Performance returned to baseline levels at 24 h. Some deaths occurred at all dose levels. In the second study, motor performance was measured after exposure to radiation alone or a drug/radiation combination ($N = 8$/group). WR-2721 was administered 30 min before exposure to 130 Gy of γ radiation from a ^{60}Co source at a dose rate of 20 Gy/min. Rats were tested on the accelerod immediately after WR-2721 treatment and at 10, 15, 30, 60, and 120 min and 24 h following radiation. Performance was significantly depressed compared to control throughout the 24 h following radiation exposure, with and without WR-2721. The decrement produced by WR-2721 and radiation alone appeared to add up to the combined drug/radiation decrement found over the 15- to 120-min test periods. In the third study assessing the effects of WR-2721 on weight, untrained rats treated with 200 or 400 mg/kg WR-2721 exhibited significant weight loss that lasted up to 3 days. Weight returned to pretreatment levels in 15 days, and no deaths occurred. In summary, the data suggest that in the rat (1) WR-2721 is behaviorally toxic at doses relevant to radioprotection, (2) WR-2721 treatment along with the stress of motor performance may combine to lower the level at which lethalities occur, (3) WR-2721 does not protect for radiation-induced PD, and (4) WR-2721 combined with radiation disrupts performance more severely than either radiation or WR-2721 alone.

INTRODUCTION

One of the most effective radioprotectants available is *S*-2-(aminopropylamino)-ethylphosphorothioic acid (WR-2721) (*1*, *2*). Survival increases in irradiated animals pretreated with WR-2721, and protection is afforded to a variety of organ systems but not the central nervous system (*3*, *4*). However, little information exists regarding the behavioral pharmacology of this drug. Behavioral changes would be a concern for individuals taking the drug during either military operations or radiation therapy.

One behavioral change of interest is the decrement in performance (PD) that occurs

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from 10 to 60 min following exposure to rapidly delivered, lethal doses of ionizing radiation (*5, 6*). A preliminary study assessing PD in monkeys performing a visual discrimination task (VDT) reported that WR-2721 offered some protection during the first 30 min after exposure to 40 Gy of radiation (*7*). However, PD developed in the next 30 min, indicating that protection lasted only a short time.[1] Other behavioral studies with animals used the conditioned taste aversion (CTA) paradigm[2] (*8, 9*). All studies reported significant aversion to saccharin-flavored water, a normally preferred substance, following pairing with either radiation or WR-2721. Cairnie (*9*) reported that CTA was greater when WR-2721 was combined with radiation, suggesting not only that WR-2721 offered no protection for this behavior, but also that its detrimental effects were additive with radiation-induced CTA.

WR-2721 is undergoing clinical testing to determine its usefulness as an adjunct in the radiation treatment of malignant tumors (*4*). However, the clinical testing indicates that WR-2721 has serious shortcomings; its side effects include nausea, vomiting, abdominal cramps, and diarrhea (*4, 10, 11*). In addition, Wagner *et al.* (*12*) reported vomiting in dogs treated with WR-2721. Thus results of a variety of research suggest that WR-2721 can produce adverse consequences that might severely disrupt behavior. The present studies were carried out to test this hypothesis by assessing the effects of WR-2721 alone and in combination with radiation on rat motor performance.

METHODS

Subjects. Subjects were 47 adult male Sprague-Dawley rats weighing 518 ± 9 g (mean ± SE). They were individually housed in clear plastic cages and maintained in keeping with the principles enunciated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council. Food and water were available *ad libitum*. Room temperature was maintained at 22°C. Relative humidity was kept at 60–70%.

Test substance. WR-2721 was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. It was dissolved in 0.9% NaCl solution (pH 7.2) immediately before ip injection at 200, 300, and 400 mg/kg body wt (administered in 1 ml/500 g). Yuhas (*3*) suggests that this range of doses has clinical applicability in humans.

Task. The accelerod apparatus and procedure have been described in detail elsewhere (*13*). Rats were trained to maintain position for as long as possible on a 2-in. diameter, gradually accelerating rod elevated 6 in. above a grid-shock floor. The rotational velocity of the accelerod increased at an average rate of 0.9 rev/s. A trial began with a subject being placed on the stationary rod, and the trial lasted until the subject fell to the grid floor below the accelerating rod. Each training session lasted from 5 to 15 min (4 to 20 trials). This range of trial durations was necessary to deal with unique intersubject requirements. It took an average of 9 training days for a rat to learn the task. In the final stages of training, shock was given only for performance that lasted less than 30 s. The task was scored as performance duration in seconds. The average performance time achieved before drug and/or radiation testing was 40 ± 2 s, with performance averaged over three trials/session/subject. Testing began with radiation and/or WR-2721 once each subject was performing stably on the accelerod at or above 25 s.

Test procedures: Performance study. Rats were injected ip in the order of 400, 300, or 200 mg/kg of WR-2721 (N 6/group), and tested immediately, at 30-min intervals for 3 h, and at 24 h after treatment. A subject served as its own control: i.e., before WR-2721 treatment, a subject was injected with an equal volume of saline and then tested as above.

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[1] R. W. Young, personal communication, 1985.

[2] B. M. Rabin, W. A. Hunt, and J. Lee, Effects of area postrema lesions on the behavioral toxicity of WR-2721 in the rat. In preparation.

We had intended to conduct the three WR-2721 iterations of 200 to 400 mg/kg and the combined radiation/WR-2721 study with the same rats in a repeat-subjects design, i.e., subjects tested in all four conditions. However, additional trained subjects were necessary because deaths occurred at all levels of WR-2721 following treatment (Table I). Of the 11 subjects used, two subjects participated in all four conditions, two were in three conditions, and one received two conditions. No systematic differences in performance were noted between the repeat- and nonrepeat-test subjects following WR-2721 or radiation/WR-2721. Iterations between treatments were conducted at an average interval of 12 days to permit clearance of WR-2721. During this interval the repeat-test subjects were run every 3 days to maintain performance.

Radiation Study. Prior to irradiation, each subject was tested for 3 days, simulating the exposure-day profile, less radiation. The Radiation Study consisted of a radiation-only group and a combined WR-2721/radiation group (N = 8/group). The combined group received 200 mg/kg of WR-2721 30 min before radiation. After irradiation, subjects were tested at 10, 15, 30, 60, and 150 min and at 24 h.

Weight Study. Rats were tested for weight change following treatment with 200 or 400 mg/kg WR-2721 (ip) for up to 20 days. Treatment and control groups existed at each dose level (N = 5/group).

Radiation. Rats were individually irradiated in a ^{60}Co facility by 1.25-MeV γ photons, right side to the source, at a midline tissue dose rate of 20 Gy/min, for a total cumulative dose of 130 Gy. This dose and dose rate were used because they had been the ED_{90} (effective dose at producing PD, 90% of the time) in a previous study (5).

Analysis. The measure of accelerod performance was time spent on the rotating cylinder. One-way analysis of variance (ANOVA) was used to assess the effects of treatment and time in the Performance and Radiation Studies (*14*). t Tests were also used to analyze the drug and radiation effects compared to control performance. A Bonferroni allocation sufficient to each ANOVA and t test was in effect to compensate for potential multiple analyses errors (*15*). In the Radiation Study, the initial test period (40 min after WR-2721 treatment or 10 min after radiation exposure) was used to assess PD. t Tests were used to analyze the Weight Study data. Chi-square analysis was applied to the lethality findings of the Performance and Weight Studies.

RESULTS

Performance Study. Accelerod performance following WR-2721 treatment is shown in Fig. 1. Performance of the WR-2721 conditions are compared against pretreatment control performance, as reflected by 0% change on the ordinate (left side). The right-side ordinate indicates the actual performance duration in seconds of the test trials.

The motor performance data were analyzed in two ways comparing performance across the eight test periods and comparing performance at each test period. In each comparison, initially one-way ANOVAs assessed performance of only the treatment groups followed by t tests comparing treatment performance against control performance. A one-way ANOVA done across the eight test periods of the treatment conditions indicated no significant difference following WR-2721. However, t tests indi-

TABLE I

Deaths Produced by WR-2721 in the Performance and Weight Studies

	Performance study		*Weight study*
Dose level (mg/kg)	*Average hour to death*	*Number of deaths*	*Number of deaths*
200	170	2/6	0/5
300	120	1/6	—
400	36	2/6	0/5

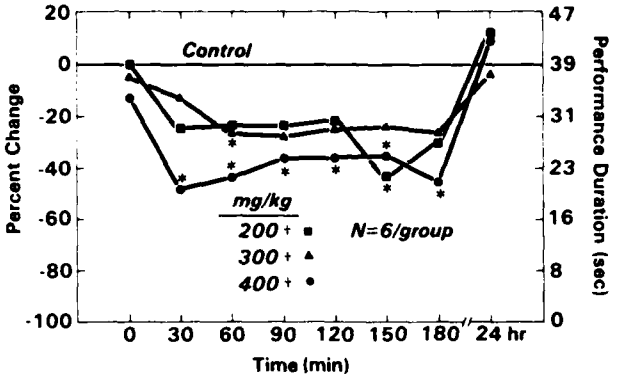

FIG. 1. Accelerod performance of rats treated with 200, 300, or 400 mg/kg WR-2721 (ip). Significant group effects over all test trials compared to control performance are shown by daggers ($P < 0.02$). Significant trial effects compared to control performance are shown by asterisks ($P < 0.02$).

cated significant differences when comparing each treatment condition over all the test periods against control performance (daggers in the figure legend). ANOVAs were done comparing performance of the three treatment groups on each of the eight test periods, but no significant differences were found. *t* Tests made on each of the eight test trials comparing treatment and control performance indicated that (a) the 400 mg/kg group was significantly below their own control performance on the 30–180 min test periods (asterisks in figure), (b) the 300 mg/kg group was significantly below control at 60 min, and (c) the 200 mg/kg performance was significantly poorer than control on the 150-min test. All treatment groups returned to control performance levels by 24 h.

Radiation Study. Figure 2 shows performance following exposure to radiation alone and after combined treatment with WR-2721 and exposure to radiation. The WR-2721 dose level in the combined condition was 200 mg/kg. The left-side ordinate indicates the percentage change from control for the dosed groups, and the right-side

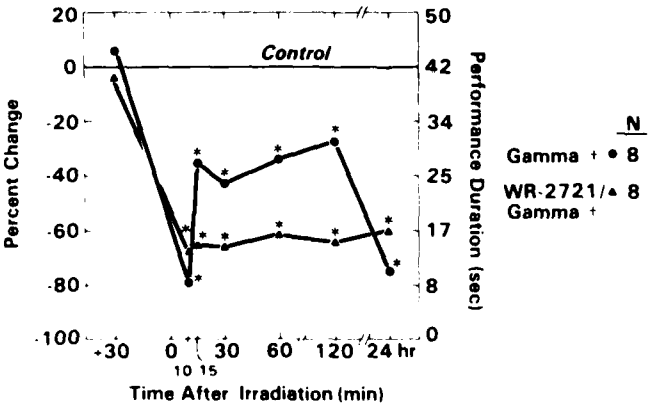

FIG. 2. Accelerod performance of rats exposed to 1.30 Gy of γ radiation or treated with 200 mg/kg (ip) of WR-2721 and then exposed to 1.30 Gy of γ radiation. Significant group effects over all test trials compared to control performance are indicated by daggers ($P < 0.02$). Significant trial effects compared to control performance are shown by asterisks ($P < 0.02$).

ordinate indicates performance time in seconds. Subjects were tested immediately after real or sham (saline) treatment with WR-2721 (+30 min time on the abscissa).

The data shown in Fig. 2 were analyzed in the same manner as the Performance Study. The radiation conditions were compared across the seven test trials. ANOVA indicated no significant differences across the test periods for the two conditions. *t* Tests comparing each radiation condition against control performance indicated a significant drop in performance across the seven test periods (daggers in the figure legend). ANOVA's compared performance at each test period for the two radiation conditions. Significant differences occurred on the 15- and the 120-min postradiation periods ($P < 0.02$). *t* Tests done on each period comparing each radiation condition to control performance indicated that both radiation conditions were significantly below control performance on all postexposure test trials, as shown by the asterisks.

No significant difference was found between the survival times of the radiation subjects (mean = 87 h, $N = 6$) and the WR-2721/radiation subjects (mean = 95 h, $N = 5$). The number of subjects assessed for lethality was smaller ($N = 11$) than the total number of subjects ($N = 16$), because subjects were excluded if the time of death was uncertain.

Weight Study. A marked weight loss occurred in all dose groups following WR-2721 treatment in the Performance Study; however, weight was not systematically studied. A Weight Study was done in rats treated with either 200 or 400 mg/kg WR-2721 or respective saline-control groups (Fig. 3). Weights were measured until they returned to pretreatment levels. In the 200 mg/kg dose and its control group, weights were measured on 6 different days (0, 1, 2, 6, 8, and 13). In the 400 mg/kg groups, weights were measured on 10 different days (0, 1, 2, 3, 6, 8, 10, 13, 15, and 20). Weight averaged 561 ± 14 g in all groups at the start of the study.

The data from the Weight Study were analyzed with *t* tests comparing percentage change from the previous test day between the treatment and respective control groups.

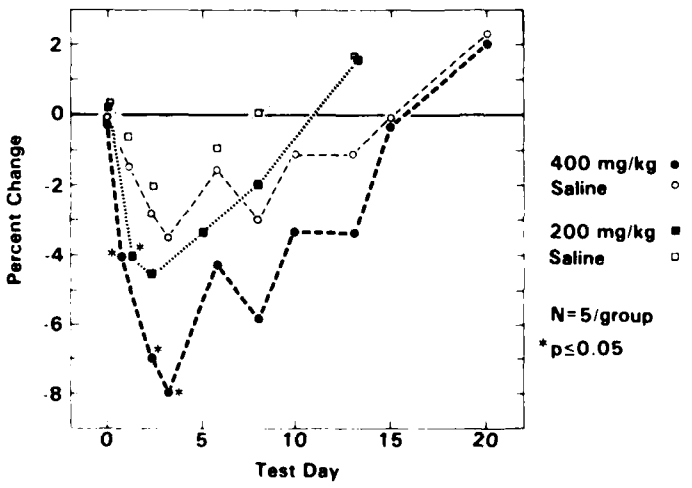

FIG. 3. Weight change of rats treated with 200 or 400 mg/kg WR-2721 (ip). Control groups existed in which rats were given an equal volume of saline. Significant effects from the respective control group were calculated based on percentage change from the previous day and are indicated by asterisks ($P < 0.05$).

Based on this analysis, the 200 mg/kg subjects lost significantly more weight than controls the day after WR-2721 treatment (asterisk at closed square). The 200 mg/kg subjects lost more weight on Day 2, but since control weights also decreased, the difference between the groups was not significant. Beyond Day 2, weight increased, returning to baseline by Day 13. The 400 mg/kg rats demonstrated a significant weight loss for 3 days after treatment (asterisks at closed circles). After Day 3, significant decreases no longer occurred and weight returned to baseline by Day 15. No significant differences existed between the treatment conditions.

Death did not occur in the Weight Study, but it did occur in the Performance Study at all dose levels (Table I). Chi-square analysis indicated a significant difference for lethality ($P < 0.05$) in the 200 and 400 mg/kg dose levels of the Performance Study (4 out of 12 subjects died) and the Weight Study (0 out of 10). No dose–response relationship existed in terms of the number of deaths in the Performance Study; however, a dose–response relationship did appear to exist in terms of the time of death; i.e., survival time decreased as dose increased (Table I).

DISCUSSION

The present study is the first to describe the effects of WR-2721 on motor performance. WR-2721 produced decrement in motor performance at doses from 200 to 400 mg/kg. The decrement was first measured 30 min after treatment, when WR-2721 offers its maximum protection against lethality (*2*). The magnitude of the decrement ranged from an average of 24% for all test periods in the 200 mg/kg dose group to an average of 44% at 400 mg/kg. The decrement lasted for the initial 3 h of testing, and recovery was complete at 24 h. In addition, marked loss of weight was noted in both the Performance and the Weight Studies. In the Weight Study, the magnitude and the duration of loss appeared to be dose dependent (Fig. 3). These studies indicate that WR-2721 significantly disrupted motor performance and produced significant weight loss at doses suggested as relevant to radioprotection in humans (*3*).

One other study reported on the effects of WR-2721 directly on performance (*7*). In this study, WR-2721 seemed to offer protection from PD in monkeys performing the VDT up to but not beyond 30 min.[1] However, implications of this study are of limited value because it was only a preliminary study and the preparation of WR-2721 in use today is different from that which was available at the time when these results were obtained. Other work also reported the adverse effects of WR-2721, but performance was not studied directly. For instance, Cairnie (*9*) and Rabin *et al.*[2] reported that WR-2721 produced a conditioned taste aversion for a normally preferred substance in rats. Coil *et al.* (*16*) argue that CTA in the rat is a parallel process to nausea and vomiting with regard to afferent pathways and central processing. Thus, since the rat cannot manifest these gastrointestinal (GI) symptoms, CTA is analogous to the GI response in other species. The CTA findings agree with human research in which GI side effects were reported. For instance, cancer patients given WR-2721 as a radiation therapy adjunct became nauseated and vomited (*10*), and normal adults in a WR-2721 safety-testing study also demonstrated abdominal cramps and diarrhea (*11*). Thus, while these GI findings imply that WR-2721 would degrade performance, prior to this study it was never tested directly, and the precise effects were unknown.

Related research from this laboratory indicates that the relationship between these GI symptoms and performance ability is not as obvious as it appears. For instance, no correlation existed between episodes of vomiting and PD in irradiated monkeys trained to perform a motor performance task (*17*). In more recent work, no correlation was noted between emesis and performance in WR-2721-treated monkeys trained to perform the VDT.[3] These studies suggest that even though it is tempting to speculate that overt signs of GI dysfunction will disrupt performance, it may be preferable to test for the performance effects directly.

The deaths produced by WR-2721 treatment alone in the Performance Study were perhaps a more striking finding here than the motor decrement (Table 1). The highest dose level used was based on the concept of maximum tolerated dose (*2*, *18*), which is usually stated as two-thirds the median lethal dose (LD_{50}). Since the rat LD_{50} for WR-2721 has been reported as 590 mg/kg (*3*), the highest dose used in the present study was 400 mg/kg. In the Performance Study, deaths occurred in all dose groups; however, no deaths occurred in the Weight Study, which suggests that lethality was produced by the added stress of accelerod performance. The added stress may have been a product of (a) motor activity, presumably because the subjects were required to work after treatment rather than rest, as is normally true, (b) an increased number of shocks delivered following treatment since performance fell below the minimal performance level of 30 s, or (c) a combination of the two. In addition, if the present synergistic effect is real and generalizable to other toxins, and if a measure of toxicity in terms of lethality is of interest beyond the resting state, the current findings imply that performance assays should be part of any initial, acute toxicity screen.

The objective of the WR-2721/radiation study was to determine if WR-2721 given 30 min prior to irradiation could mitigate PD assessed 10 min later. WR-2721's potential as a prophylactic for lethality was reported over 20 years ago, and considerable research has been conducted during that time; however, only one study dealt with PD (*7*). The present data indicate that WR-2721 offered no protection from radiation-induced motor performance PD for the rat. As shown in Fig. 2, the combined condition performance 40 min after WR-2721 (10 min after radiation) was the same as the radiation-only condition, i.e., 70 to 80% below baseline. While the WR-2721/radiation subjects remained 60% below baseline for the entire 24-h test period, the radiation condition showed some recovery, to 30–40% of baseline at the 15-min test period. Further, testing beyond 24 h was not conducted in the radiation iterations even though the subjects lived another 3 days because the subjects were so debilitated.

Another interesting point about performance effects can be made by comparing the radiation data in Fig. 2 with the 200 mg/kg WR-2721 data in Fig. 1. The overall post-treatment decrement of the WR-2721 subjects in Fig. 1 and the postradiation decrement of the radiation-only subjects (Fig. 2) equal the postradiation decrement of the combined condition (Fig. 2), i.e., 20% (WR-2721) plus 40% (radiation) equals 60% (combined condition). Cairnie (*9*) reported a similar finding when assessing the effects of WR-2721 and radiation on the CTA paradigm. The important point in the present radiation study was that rather than act as a protectant, the combination of WR-2721 and radiation made performance worse than either condition alone.

[3] V. Bogo, research in progress.

No difference existed in the survival times of the radiation subjects and the WR-2721/radiation subjects; thus WR-2721 did not protect against lethality, one of its stated benefits (*2*). The lethality finding was not unexpected, since the aim of this study was to determine if WR-2721 could protect against PD. Previous work in our laboratory showed that 130 Gy was the 90% PD-effect level for accelerod-trained subjects exposed to γ radiation (*5*). The maximum dose reduction factor for lethality offered by WR-2721 is reported as 2.7 in the mouse (*2*). Since the rat $LD_{50/30}$ is about 6 Gy (*19*), the level of radiation used here was well above any potential protection from lethality that WR-2721 could offer. We plan to conduct a study assessing motor performance changes following a sublethal dose of radiation in the rat to look at the lethality–prophylactic ability of WR-2721. In the present study, while the WR-2721/radiation combination did not extend life, at least WR-2721 combined with radiation did not kill rats sooner than radiation alone, making the situation worse as was the case with the Performance Study.

ACKNOWLEDGMENTS

The authors thank C. A. Boward and G. G. Kessell for their expert technical assistance in training and testing the subjects. We also thank E. J. Golightly for the exposure of the subjects. This research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency (DNA), under research work unit MJ 00096. Views presented in this paper are those of the authors; no endorsement by DNA has been given or should be inferred.

RECEIVED: February 25, 1985; REVISED: June 25, 1985; RE-REVISED: July 16, 1985

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Reprinted from American College of Surgeons 1985 Surgical Forum Volume XXXVI.

COMPARISON OF THE EFFECTS OF SOLUBLE AND PARTICULATE FORMS OF GLUCAN, AN IMMUNOMODULATOR, ON PROSTAGLANDIN SYNTHESIS BY RAT PERITONEAL MACROPHAGES

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GLUCAN, AN IMMUNOMODULATOR derived from the yeast *Saccharomyces cervesiae*, exists in two preparations, particulate (glu-P) and soluble (glu-F). Both preparations enhance host antibacterial and antineoplastic resistance (2). Unlike glu-F, glu-P is associated with granulomatous reactions within the reticuloendothelial system (RES) (3) and endotoxin sensitivity (1). Since some of the adverse effects of glu-P may be mediated by prostaglandins (PGs), we compared the effects of glu-P and glu-F on PG production by rat peritoneal macrophages (PMøs).

MATERIALS AND METHODS

Resident PMøs were incubated (10^6 cells/well) in minimum essential medium (MEM) at 37°C. After two hours, cells were washed, then stimulated for five hours with various concentrations of glu-P or glu-F in MEM (1.0 mL/well). Concentrations of immunoreactive (i) thromboxane (Tx) B_2 (stable metabolite of TxA_2) and i6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) were measured in culture supernatants by radioimmunoassay. Protein content of the PMø monolayer was determined using Bio-Rad. Prostanoid concentrations per microgram of protein were calculated, and the results were expressed as mean percentage (%) change ± standard error vs control (ie, no glucan). Differences between the groups were assessed by two-way analysis of variance, with drug and dose as independent sources of variation.

RESULTS

Glucan stimulation did not alter viability of the PMøs (trypan blue exclusion). Glucan P and F stimulated synthesis of PGs. As shown in Table 1, $iTxB_2$ increased with increasing doses of both drugs, glu-P being the stronger agonist. The concentration of i6-keto-$PGF_{1\alpha}$ in supernatants

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Table 1

PG	Glucan	Glucan dose (μg/mL)*				Statistics
		0.1	1.0	50	100	
TxB_2	F	60 ± 9	116 ± 20	337 ± 28	377 ± 30	Dose: $F_{3,72} = 26.8$, $P < 0.001$
	P	141 ± 17	212 ± 5	1,722 ± 265	2,010 ± 341	Drug: $F_{1,72} = 52.7$, $P < 0.001$
6-keto-$PGF_{1\alpha}$	F	150 ± 23	255 ± 28	412 ± 69	393 ± 59	Dose: $F_{3,72} = 21.6$, $P < 0.001$
	P	131 ± 15	181 ± 12	407 ± 24	473 ± 66	Drug: $F_{1,72} = 0.02$, $P < 0.05$

*N = 10.

also increased with larger glucan doses, but there was no difference in the effect of the two preparations.

DISCUSSION

Glucan-P, but not glu-F, evokes granuloma formation within the major RES organs, consisting of hypertrophic and hyperplastic macrophages (Møs) (2,3). Cotreatment with indomethacin attenuates glu-P-induced granulomogenesis while not interfering with ingestion of glu-P by Møs (3). These data suggest a role for PGs in the pathogenesis of glu-P-induced granulomas. We showed PG release by glucan-stimulated PMøs depends on the preparation of glucan employed. This observation may help explain why glu-F does not induce granuloma formation.

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Enhanced Activity of the Macrophage-Like Cell Line J774.1 Following Exposure to Gamma Radiation

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Exposure of the macrophage-like cell line J774.1 to 20 gray of cobalt-60 gamma radiation resulted in a block of tritiated thymidine incorporation, along with an increase in cell "activation," as assessed by increases in lysosomal enzyme and ectoenzyme content, PMA-induced H_2O_2 production, and NBT staining, ingestion of E(IgG), spreading, and membrane ruffling. These changes are evident within 1 day postradiation and peak at 4 days postradiation.

Key words: gamma radiation, macrophage, J774.1 cells, hydrogen peroxide

INTRODUCTION

It is well known that macrophages can exist in various states of activation, as assessed by their secretory capacity, oxidative burst in response to stimuli, or cytotoxic ability [9]. Their state of activation is influenced by in vivo factors, and it can also be modulated in vitro by exposure to a variety of stimuli. Both lipopolysaccharides (LPS) and gamma interferon enhance macrophage activation, by priming cells so that their oxidative response to subsequent stimuli is increased [8,11], whereas tumor-cell-conditioned medium decreases the production of oxidative products by macrophages [16].

Just as with primary macrophages, the properties of some macrophage-like cell lines can be modified by changes in growth conditions or by the addition of stimulatory factors [4,5,17]. For example, the macrophage-like cell line J774 derived from a murine reticulum sarcoma is much more cytotoxic when grown as an ascites tumor than when cultured in vitro [14], and addition of lipopolysaccharide to the culture medium increases the release of interleukin 1 and prostaglandin E_2 by J774 cells [6,10,15].

Received October 2, 1984; accepted February 12, 1985.

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In this paper we report that exposure of J774.1 cells to doses of gamma radiation that inhibit the incorporation of thymidine results in an increase in activation, as assessed by a variety of functional parameters, including an increase in H_2O_2 release in response to phorbol myristate acetate. Radiation may therefore be a useful tool for studying J774 cells in different states of activation.

MATERIALS AND METHODS

Cells

J774.1 cells obtained from the American Type Culture Collection were maintained in spinner culture in tissue culture medium (TCM) containing RPMI-1640 (Flow Labs, McLean, VA), supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin (DIFCO Labs, Detroit, MI), 0.03% L-glutamine (Sigma, St. Louis, MO), and 5% fetal bovine serum (HyClone Labs, Logan, UT). Cells to be used in experiments were either cultured in Teflon dishes so that they could be harvested easily or plated on tissue culture dishes. Control cells and irradiated cells were plated at the same concentrations and were maintained under identical conditions in a 37°C 5% CO_2–95% air incubator for the duration of the studies. Irradiation was performed bilaterally using a cobalt-60 radiation source (5 gray/min) on cells 1 h after plating on tissue culture dishes or cells suspended in Teflon jars.

^{3}H-Thymidine Uptake

Cells were washed twice with phosphate-buffered saline containing calcium and magnesium (PBS) (GIBCO, Grand Island, NY), and incubated for 1 h in TCM containing 5 μC/ml ^{3}H-thymidine. They were then washed three times with PBS, incubated for 1 h in TCM containing 0.1 mM cold thymidine to remove nonspecifically bound ^{3}H-thymidine, and washed three times with PBS. The washed cells were lysed with 0.2% Triton for 1 h, and the cell lysate was assayed for tritium and protein. Results were expressed as ^{3}H uptake per milligram of cell protein.

Cell Protein and Adherence

Control and irradiated cells were cultured in Teflon jars at approximately 0.5×10^6 cells/ml. On days 2 and 4 postradiation, cells were harvested and counted, and the cell protein was determined. Proteins were determined by the Bradford method using a Bio-Rad assay kit (Rockville Centre, NY) with bovine plasma albumin as the standard. Data were expressed as protein per cell.

The ability of cells to adhere to tissue culture dishes was assessed by plating cells grown in Teflon dishes on tissue culture plates for 1 h, after which time the dishes were washed twice with PBS to remove non-adherent cells. Adherent cells were lysed with 0.2% Triton, and the protein in the cell lysate was measured. The total protein in the cell suspension initially added to the dishes was measured, and the percentage of total protein remaining adherent to the culture dish was determined.

Phagocytosis

Bovine red blood cells (E) were opsonized with a subagglutinating titer of antibovine red blood cell IgG (Cappel Laboratories, Cochranville, PA). E(IgG) were incubated with sodium chromate Cr-51 (New England Nuclear, Boston, MA) in RPMI 1640 at approximately 250 μCl/10^9 E(IgG) for 1 h at 37°C, followed by four washes with RPMI 1640 to remove the free Cr-51.

Cr-51-labelled E(IgG) were added to adherent J774.1 cells at a 100:1 ratio of E(IgG) to J774.1. The number of J774.1 cells per dish was estimated by counting the number of cells in three different low-power fields. Dishes were incubated for 1 h at 37°C in a 5% CO_2–95% air atmosphere, at which time cells were washed with PBS and hypotonically lysed with 0.2% NaCl for 30 sec to lyse all unphagocytized Cr-51 E(IgG). Plates were then washed twice with PBS, cells were lysed with 1 ml of 0.2% Triton X-100 for 1 h, and cell lysates were counted. Paired dishes not fed E(IgG) were used to determine the protein content in each experimental group. The Cr-51 content per E(IgG) was determined, and data were expressed either in terms of E(IgG)/cell or E(IgG)/mg protein.

Enzyme Assays

β-Glucuronidase levels were determined by measuring the change in absorbance following the conversion of phenophthalein glucuronic acid to phenophthalein and glucuronic acid. Levels of β-glucuronidase were measured from cell lysates obtained from a 1-h incubation in 0.2% Triton X-100 and expressed as nM of substrate turnover per milligram of cell protein per hour.

The ectoenzymes 5′-nucleotidase, alkaline phosphodiesterase, and leucine aminopeptidase were assayed as previously described [7]. Samples for assays were prepared by lysing adherent cells with 0.05% Triton X-100 for 15 min at 4°C. Catalase activity was measured on cell fractions obtained by scraping off adherent cells and sonicating them on ice for 40 sec. Cell sonicates were then assayed for their ability to break down H_2O_2 by following the destruction of H_2O_2 spectrophotometrically at 230 nm [1]. Lysozyme levels were measured spectrophotometrically by measuring the lysis of Micrococcus lysodiekticus. Lactic dehydrogenase (LDH) levels were assayed spectrophotometrically by following the consumption of NADH during the conversion of pyruvate to lactate. The LDH released into the medium was expressed as a percentage of the total cellular LDH.

Nitro-Blue Tetrazolium Staining

Cells were placed in PBS containing 10 mM glucose. One μg/ml of PMA was added to half the dishes. After a 1-h incubation at 37°C, nitroblue tetrazolium (NBT) was added to the dishes to give a final concentration of 0.05%. Following a 30-min incubation at 37°C, dishes were washed three times with PBS and fixed with Diff-Quik fixative (Harleco, Gibbstown, NJ). Cells were observed at 250 × and scored positive if they contained blue grains.

H_2O_2 Assay

J774.1 cells adherent to tissue culture dishes were washed twice with PBS and exposed to 2 ml of reaction mixture containing PBS, 10 mM D-glucose, 6 purpurogallin units/ml of horseradish peroxidase (Sigma type II), and varying amounts (5–40 nmoles/ml) of scopoletin (Sigma, St. Louis, MO). Concentrations of scopoletin were selected so that $\leqslant$ 60% was oxidized. Phorbol myristate acetate (PMA) at 1 μg/ml (Consolidated Midland Corp., Brewster, NY) was added to half the cultures. Cells were incubated at 37°C in 5% CO_2/95% air for 1 h, at which time the reaction mixture was removed and placed in clean test tubes. H_2O_2 release was determined by measuring percent decrease in fluorescence using PBS and the reaction mixture to set the 0% and 100% fluorescence, respectively. A standard curve was obtained by adding known amounts of ethyl-H_2O_2 (Polysciences, Warrington, PA) to the reaction

mixture and observing the subsequent decrease in fluorescence. In some studies Escherichia coli lipopolysaccharide (LPS) (List Biological, Campbell, CA) was added to cell cultures 12 h prior to the H_2O_2 assay.

RESULTS

Tritiated Thymidine Uptake

The effect of radiation on the replicating ability of J774 cells was assayed indirectly by measuring the uptake of thymidine. Adherent J774 cells were exposed to varying doses of radiation, and ^{3}H-thymidine uptake was measured at 2, 3, and 6 days postradiation. Figure 1 shows thymidine uptake data from cells at 2 days postradiation as a function of radiation dose. The data, expressed as percentage of thymidine uptake of nonirradiated cells, demonstrate that increasing doses of radiation progressively decreased the uptake of tritiated thymidine. At doses of 20 gray or greater, the uptake of thymidine was reduced to background levels, indicating that DNA synthesis was negligible. Similar data were obtained on days 3 and 6 postradiation, indicating that the block in DNA synthesis was irreversible. The radiation dose of 20 gray was chosen for most of the subsequent experiments since it was the lowest dose of radiation tested that completely blocked thymidine uptake.

Cell Survival

To determine whether exposure to 20 gray killed a significant number of cells, the percentages of total LDH released into the supernatant in cultures of irradiated cells and control cells were measured at 1 and 2 days postradiation. Between 8 and 13% of the total LDH was released into the supernatant in both control and radiated dishes on days 1 and 2 postradiation, indicating that exposure to 20 gray did not produce a significant increase in cell death. In addition, cell counts determined over 4 days postradiation indicated that the number of cells in the cultures was stable. Irradiated cultures could be maintained for 2 wk or longer, and although cell counts were not done over this time period, cultures appeared stable and healthy.

Cell Size, Morphology, and Adherence

The change in cell size following exposure to 20 gray was determined indirectly by measuring the protein content and the cell numbers of both irradiated and control cells cultured in Teflon jars. As shown in Figure 2, the protein/cell for irradiated cells increased significantly with time, so that by day 6 postradiation, irradiated cells had 2.5 times more protein/cell than did control cells.

In addition to being larger than control cells, irradiated cells were morphologically distinct (Fig. 3). Two days after exposure to 20 gray, cells were more spread and vacuolated than were control cells. In addition, their membranes were much more ruffled. The irradiated cells maintain this appearance for the duration of the culture period (2 wk or longer).

The ability of cells to adhere to tissue culture dishes was examined by culturing irradiated cells and control cells in Teflon jars for both 2 and 6 days and then plating them on tissue culture dishes. Following a 1-h incubation, dishes were washed vigorously and the adherent protein measured. Greater than 90% of the total cellular protein added to the dishes remained following vigorous washing in both control and irradiated cells, indicating that the adherence of irradiated cells was indistinguishable from control cells at 2 and 6 days postradiation.

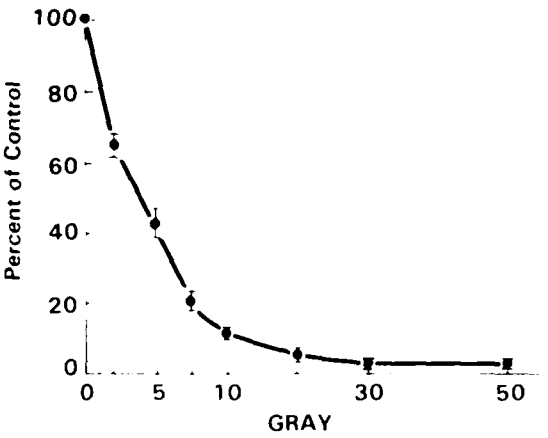

Fig. 1. Uptake of ^{3}H-thymidine as a function of radiation dose. Irradiation was performed 2 days before thymidine uptake. Data are expressed as percentage of thymidine uptake of control cells (nonirradiated), and represent mean ± SEM of 10 measurements.

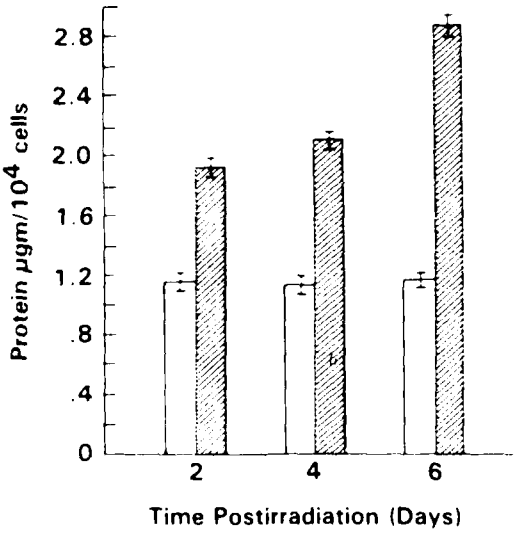

Fig. 2. Protein content/cell for control cells (open bars) and irradiated cells (shaded bars) at 2, 4, and 6 days postradiation. Data represent mean ± SEM of 12 different measurements.

Enzyme Content

The cellular levels of β-glucuronidase, catalase, and the ectoenzymes, 5′-nucleotidase, alkaline phosphodiesterase (APD), and leucine amino peptidase (LAP) were measured in both control and radiated J774.1 cells at 2 and 4 days after exposure to 20 gray. The level of lysozyme released into the supernatant was also monitored. The data expressed in terms of cell protein are summarized in Table 1; they indicate that radiation exposure significantly ($P > .01$) increased the β-glucuronidase, APD,

Fig. 3. Phase contrast photomicrograph of (a) control and (b) irradiated (20 gray) cells at 2 days postradiation.

TABLE 1. Effect of Radiation on Enzyme Content

	Day 2		Day 4	
	Control	20 Gray	Control	20 Gray
β-Glucuronidase[a] (nM/h/mg protein)	533 ± 66	1,024 ± ±25	592 ± 34	1,214 ± 73
Lysozyme[b] (μg/mg protein)	9.6 ± 3.4	20.8 ± 2.3	11.3 ± 1.2	35.1 ± 3.6
Catalase[a] (U/mg protein)	1.6 ± 0.07	2.6 ± 0.1	1.7 ± 0.06	2.9 ± 0.1
Alkaline phosphodiesterase[a] (nmoles/min/mg protein)	3.4 ± 0.7	7.0 ± 0.9	4.7 ± 0.6	9.7 ± 1.6
Leucine amino peptidase[a] (nmoles/min/mg protein)	42.1 ± 6	62.5 ± 8	51.7 ± 7	85.5 ± 13

[a]Cellular content. Mean ± SE of 12 to 15 different measurements.
[b]Lysozyme accumulated in supernatant during indicated culture period. Supernatant harvested and measured at 48-h time periods. Mean ± SE of 20 measurements.

LAP, and catalase content and the release of lysozyme by J774.1 cells. 5′-Nucleotidase was undetectable in both control and irradiated cultures. Lysozyme release over the first 2 days postradiation doubled and then tripled by day 4. The cellular levels of β-glucuronidase, APD, and catalase content in the cells also doubled at day 2 but showed little or no additional increase on day 4. The LAP levels increased by 66% on day 2 and showed no additional increase on day 4.

In order to determine the relationship between radiation dose and the increase in enzyme content, cells were exposed to varying doses of gamma radiation and cellular β-glucuronidase levels measured on days 2 and 4 postradiation (Fig. 4). There was a dose-dependent relationship between the increase in β-glucuronidase and radiation, with doses of radiation as low as 2.5 gray having a significant effect. The β-glucuronidase content of the cells exposed to 20 and 30 gray was slightly higher ($P \leq .05$) on day 4 than on day 2, but the levels of β-glucuronidase peaked at the same radiation dose (20 gray) on both days.

Phagocytosis

Initially the ability of both irradiated and control J774.1 cells to ingest E(IgG) was assessed visually on adherent cultures. Greater than 95% of the cells in both cultures were phagocytic. To quantitate more accurately the number of E(IgG) ingested by both groups of cells, the uptake of Cr-51-labeled E(IgG) was measured. The time courses of the uptake of E(IgG) in both control cells and cells exposed to 20 gray 4 days before are shown in Figure 5. Both groups of cells were plated 4 days before the assay at the same density and fed every 2 days. The data are expressed in terms of E(IgG)/cell and in E(IgG)/mg cell protein. The uptake of E(IgG) plateaued by 30 min for both control and irradiated cells. Irradiated cells ingested more than four times as many E(IgG)/cell as did control cells (Fig. 5a). When the data were expressed in terms of cell protein, the irradiated cells still showed significantly more phagocytosis than did the control cells, although the difference between the groups was slightly reduced (Fig. 5b). Therefore, even when the increase in cell size is taken into account by expressing the data in terms of cell protein, the irradiated cells ingested more E(IgG) than did the control cells.

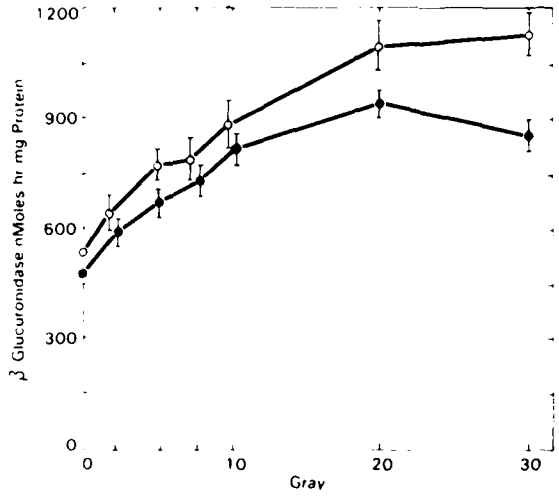

Fig. 4. β-Glucuronidase content of cells as a function of radiation dose. Measurements were made within 2 (●) or 4 (○) days postradiation. Data are mean ± SEM of eight different measurements.

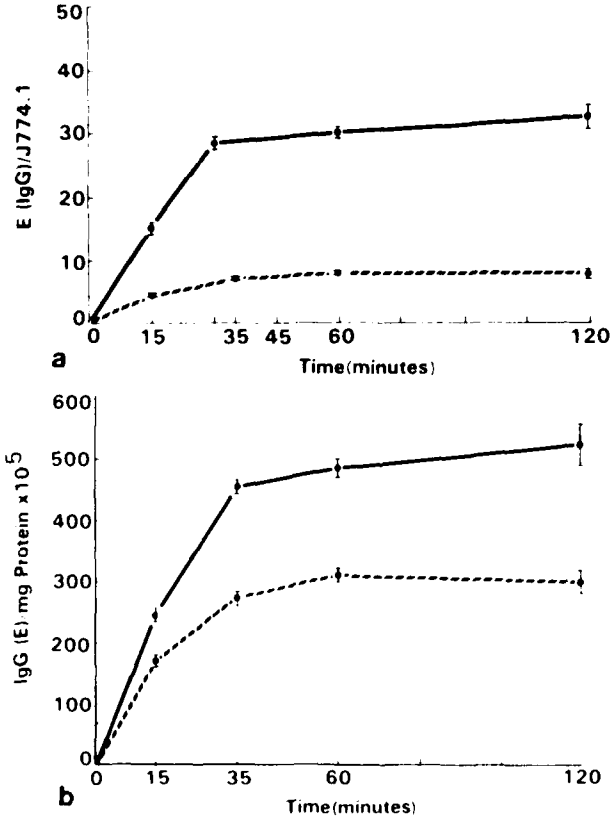

Fig. 5. Time course of uptake of E(IgG) by control cells (dashed line) and irradiated cells (20 gray) (solid line) at 4 days postradiation. E(IgG):J774.1, ratio 100:1. Mean ± SEM of 6 measurements. a) Data are expressed E(IgG)/cell; b) Same experiment as in a, but data are expressed as E(IgG)/mg protein.

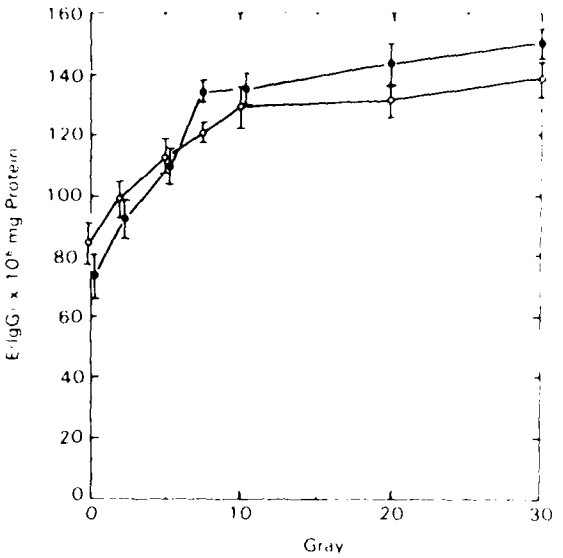

Fig. 6. Uptake of E(IgG) as a function of radiation dose at 2 (○) and 4 (●) days postradiation. Uptake was measured at 1 h. Data are mean ± SEM of 12 different measurements from four experiments.

The effects of varying doses of radiation on the ability of J774.1 cells to ingest E(IgG) were also studied. As shown in Figure 6, the phagocytic ability of the cells increased with increasing radiation doses, up to 7.5 gray. The ingestion of E(IgG) plateaued at doses of radiation equal to or greater than 7.5 gray. The dose-response curves on days 2 and 4 postradiation did not differ significantly, except that on day 4 cells exposed to 7.5 gray ingested slightly more E(IgG) ($P \leqslant .05$) than on day 2.

H_2O_2 Release

Considerable variation was seen from experiment to experiment in the level of H_2O_2 generated by both control and irradiated (20 gray) cells in response to PMA. In spite of the variability, irradiated cells consistently released more H_2O_2/mg cell protein in response to PMA than did the control cells. The level of H_2O_2 generated in five different experiments ranged from 24 to 180 nmoles/h/mg protein with a mean of 75 ± 13 for control J774.1 cells, compared to 47 to 429 nmoles/h/mg protein with a mean of 183 ± 24 for cells exposed to 20 gray of gamma radiation 4 days prior to the assay.

In order to control the possibility that the nonirradiated (dividing) cells were depleting the medium of nutrients required to sustain an oxidative burst faster than were the irradiated (nondividing) cells, a series of experiments were done in which control cells were plated at half the density of cells to be irradiated, and all cells were refed at 6-h and 12-h intervals. Similar differences in PMA-induced H_2O_2 production were seen in these studies (data not shown). The addition of catalase (66 μg/ml) to the reaction mixture reduced H_2O_2 production by > 90%, indicating that the measured decrease in fluorescence was dependent on the presence of H_2O_2. The time course of the radiation-induced increase in H_2O_2 production in response to PMA is shown in Figure 7. By day 1 postradiation there was a significant increase in H_2O_2 production ($P > .01$), which further increased and plateaued at days 3 and 4. There

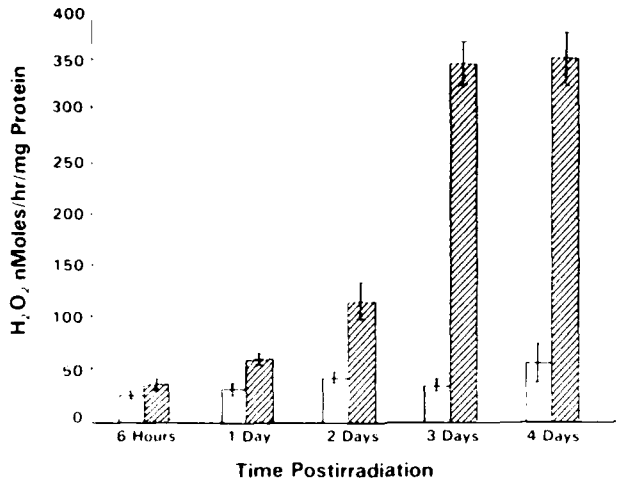

Fig. 7. H_2O_2 production in response to a 1-h exposure to PMA (1 μg/ml) by control cells (open bars) and irradiated (20 gray) cells (shaded bars) at various times following radiation exposure. Data are mean ± SEM of five measurements.

was no H_2O_2 release from either irradiated or control cells in the absence of PMA at any of the times measured in Figure 7.

Experiments were performed in which PMA-induced H_2O_2 release was assayed in both irradiated and control cells exposed to LPS for 12 h prior to the addition of PMA. These experiments were designed to investigate whether LPS, which is known to stimulate J774.1 cells [6,10] could further enhance H_2O_2 production in irradiated cells. There was a significant increase in the PMA-induced release of H_2O_2 from unirradiated cells exposed to LPS (1 μg/ml) when compared to cells grown in the absence of LPS (30.2 ± 3 nmoles/h/mg protein for control cells versus 46.8 ± 6 nmoles/h/mg protein for LPS treated cells). However, the addition of LPS to irradiated cells (2 days postradiation) for 12 h prior to the addition of PMA produced no enhancement in H_2O_2 release (121.8 ± 17 nmoles/h/mg protein for untreated cells vs. 129.5 ± 13 nmoles/h/mg protein for LPS treated cells).

NBT Staining

The percentage of NBT-positive cells in both control and irradiated cultures treated with PMA (1 μg/ml) for 1½ h was determined at 6 h and at 2 and 4 days postradiation. As shown in Figure 8, control cells incubated without PMA exhibited no NBT staining, while 5–10% of the control cells exposed to PMA stained positively. Six hours after radiation exposure, no difference was seen between the irradiated and control groups. However, on day 2, there were many more NBT-positive cells in irradiated cultures exposed to PMA than in paired control cultures. By day 4, the number of NBT-positive cells in the irradiated PMA-treated cultures increased to 70%. However, by day 4, the percent of NBT-positive cells in irradiated cultures without PMA significantly increased.

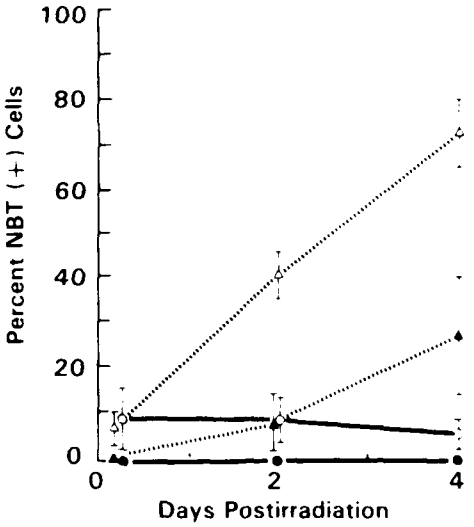

Fig. 8. Percentage of NBT + cells in cultures at 6 h and at 2 and 4 days postradiation. Control cells (solid line) with (○) and without (●) PMA, 1 μg/ml. Irradiated cells (20 gray) (dashed line) with (△) and without (▲) PMA, 1 μg/ml. Mean ± SEM of eight measurements.

DISCUSSION

The murine cell line J774 has been studied extensively as a source of large numbers of relatively homogenous macrophage-like cells [13]. In addition, the isolation of a number of different J774 clones varying in functional characteristics has made these cells particularly useful [2,18]. This paper demonstrates that the functional state of the parent cell line can be modulated by exposure to gamma radiation. Gamma radiation (20 gray) blocks thymidine incorporation and increases lysosomal enzyme content, ingestion of E(IgG), and PMA-stimulated oxidative response in these cells without producing significant cell death. These changes were evident at both 2 and 4 days postradiation, and are consistent with an increase in the state of activation of the cells [9]. Decreases in the ectoenzymes 5′-nucleotidase and alkaline phosphodiesterase are associated with anti-tumor activity in mouse peritoneal macrophages [7]. In our studies, the level of 5′-nucleotidase was undetectable in both control and radiated J774.1 cells, but alkaline phosphodiesterase levels increased postradiation, indicating that postradiation changes may not be associated with increases in cytotoxicity.

It is well known that blocking cell division by a variety of means, including radiation, can stimulate differentiation in a variety of poorly differentiated tumor cell lines [12]. Although J774.1 cells have many of the characteristics of mature macrophages and are therefore reasonably well differentiated, radiation results in enhancement of some of these functions. J774 cells can also be modulated by LPS, a well-known immunopotentiator, which increases the release of interleukin 1 and prostaglandins by the cells [6,10]. Interestingly, LPS inhibits the growth of J774 cells [6,10,15]. Takasaki and Leive [17] have recently shown that the activity of another well-characterized macrophage-like cell line, P388D1 cells, can be modulated by

exposure to DMSO. P388D1 cells bind but do not normally ingest IgG-coated particles. However, when grown in DMSO for 1 day or more, they ingested E(IgG). Although DMSO slowed the growth of these cells, the stimulation of Fc-mediated ingestion preceded the growth-inhibiting effect. In our studies, exposure to 20 gray of cobalt-60 blocked tritiated thymidine uptake so that there was no increase in cell number during the time that the functional changes in the J774.1 cells were noted. The dose dependency of the increase in β-glucuronidase content peaked at 20 gray, the radiation dose that completely blocked thymidine incorporation. This supports the view that the blocking of cell division produced by radiation resulted in the functional changes. However, the dose-response curve for the increased phagocytosis peaked at 7.5 gray, indicating that the situation was more complicated. We have recently shown that the ingestion of E(IgG) by mouse thioglycollate-induced peritoneal macrophages decreased by 4 days after exposure to 7.5 gray of gamma radiation [3]. It is possible that the peak in the dose response of ingestion at 7.5 gray resulted from the sum of two radiation effects: one that enhanced ingestion and one that inhibited it.

Both H_2O_2 measurements and NBT staining indicated that the control J774.1 cells used in this study showed a poor oxidative response to PMA. Those findings agree with the observation of Damiani et al [2] that the majority of J774 cells lacked the ability to produce a significant respiratory burst. However, following radiation exposure, H_2O_2 production and the number of NBT-positive cells in response to PMA increased significantly with time postradiation. The increased H_2O_2 production in response to PMA in irradiated cells was not due to a change in the ability of cells to break down H_2O_2, since cell fractionates isolated from irradiated cells contained more catalase activity than did cell fractionates of control cells. Exposure of irradiated cells to LPS for 12 h prior to PMA stimulation produced no additional increase in H_2O_2 production while it did enhance H_2O_2 production in control cells. The lack of additivity between the effects of LPS and irradiation on H_2O_2 production may be due to the fact that irradiated J774 cells are already maximally stimulated.

The increase in H_2O_2 released by irradiated cells in response to PMA peaked at days 3 and 4 postradiation. A concomitant increase was seen in the number of NBT-positive cells in irradiated cultures exposed to PMA. However, a significant number of cells in the irradiated cultures not exposed to PMA stained positive for NBT on day 4 postradiation. We do not know why the irradiated J774.1 cells showed an increase in spontaneous NBT reduction with time. But it is likely that this increase was not related to an increase in reactive oxygen metabolites because a concomitant increase in H_2O_2 production was not noted.

The increases in H_2O_2 production, NBT staining, ingestion of E(IgG), and enzyme content in the irradiated cell were associated with an increase in membrane ruffling and cell spreading. These changes, evident 2 to 4 days postradiation, are characteristics often associated with more activated macrophages [9]. Therefore, radiation may be a useful tool in modulating the activity of these cells.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Michelle D'Alesandro for performing the ectoenzyme assays and to Ms. Karen Schweitzer for excellent technical assistance.

This work was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00020. The views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

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Exp. Hematol. 13:532–538 (1985)

Experimental **Hematology**

Cell Kinetics of GM-CFC in the Steady State

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(Received 4 May 1984; accepted 26 November 1984)

Abstract. The kinetics of cell turnover for myeloid/monocyte cells that form colonies in agar (GM-CFC) were measured through the progressive increase in their sensitivity to 313-nm light during a period of cell labeling with BrdCyd. Two components of cell killing with distinctly separate labeling kinetics revealed both the presence of two generations within the GM-CFC compartment and the properties of the kinetics of the precursors of the GM-CFC. These precursors of the GM-CFC were not assayable in a routine GM-CFC assay when pregnant mouse uterus extract and mouse L-cell-conditioned medium were used to stimulate colony formation but were revealed by the labeling kinetics of the assayable GM-CFC. Further, these precursor cells appeared to enter the assayable GM-CFC population from a noncycling state. This was evidenced by the failure of the majority of these cells to incorporate BrdCyd during five days of infusion. The half-time for cell turnover within this precursor compartment was measured to be approximately 5.5 days. Further, these normally noncycling cells proliferated rapidly in response to endotoxin. High-proliferative-potential colony-forming cells (HPP-CFC) were tested as a candidate for this precursor population. The results of the determination of the kinetics for these cells showed that the HPP-CFC exist largely in a G_0 state, exiting at an average rate of once every four days. The slow turnover time for these cells and their response to endotoxin challenge are consistent with a close relationship between the HPP-CFC and the G_0 pool of cells that is the direct precursor of the GM-CFC.

Key words: HPP-CFC — GM-CFC — BrdUrd — Kinetics

Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

Research was conducted according to the principles enunciated in the "Guide to the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

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Upon their isolation from normal murine bone marrow, myeloid/monocytic cells that form colonies in agar (GM-CFC) represent a heterogeneous population [1–3]. These heterogeneities can be detected through physical properties of the cells [1, 2], differences in membrane receptors [4- 6], or the relative porportion of cells in the various phases of the cell cycle [7, 10, 14]. The separation of GM-CFC subpopulations based on buoyant density [2, 8], sedimentation velocity [1], S-phase content [7, 10], or their toxic response to specific drugs [7, 9] has led several workers to conclude that the basis for the hetrogeneity in GM-CFC may be either the proliferative future of the cell or the state of differentiation [11–13]. As an example, colony size, which is presumably related to the proliferative future of the colony-forming cell, has been recently shown to identify a relatively distinct population of the GM-CFC [9, 13]. Using this discriminator, Bradley and co-workers identified a minor GM-CFC subpopulation, the "high-proliferative-potential colony-forming unit" (HPP-CFC), which appears to be a candidate precursor for the majority of the GM-CFC [9, 13]. These cells, optimally expressed as colonies only in the presence of human serum and spleen-conditioned medium, are largely resistant to S-phase-specific cytotoxic agents [9, 13, 15].

In recent work, while measuring the cell-cycle kinetics of the GM-CFC under conditions that limited the expression of HPP-CFC, we observed a subpopulation of GM-CFC that appeared to have recently come from a pool of noncycling cells [14]. Since this transition is likely to be important to the understanding of the relationship between the HPP-CFC and the general GM-CFC population, we have examined in detail the BrdCyd-labeling kinetics of the cell populations involved.

To examine these kinetics, the changes in sensitivity of GM-CFC and HPP-CFC to 313-nm light were measured over an extended period of BrdCyd infusion. The kinetics for the development of 313-nm light sensitivity for initially ultraviolet (UV) resistant GM-CFC are consistent with these cells having recently entered the cell cycle from a pool of G_0 cells. In addition, the turnover time for this pool of

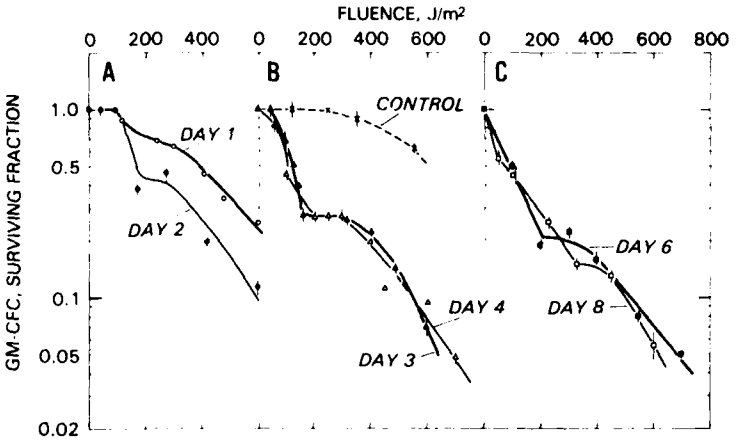

Fig. 1. GM-CFC survival after BrdCyd 313-nm-light treatment. After BrdCyd infusion for the period shown, cell suspensions of GM-CFC were exposed to the fluence of 313-nm light indicated. Control cultures were derived from animals not receiving BrdCyd. *Error bars* representing ± 1 SE are shown when they are larger than the symbol. Data represent the mean of triplicate samples.

G_0 cells and their response to endotoxin challenge are consistent with a close relationship between the cells within this G_0 pool and the HPP-CFC [9, 13].

Material and methods

Mice. B6D2F_1 female mice, 12–16 weeks of age (Jackson Laboratory, Bar Harbor, ME), were used throughout. Control and experimental mice, randomized with respect to age, were maintained on a 6:00 AM to 6:00 PM light–dark cycle. Wayne Lab-Blox and hyperchlorinated water were available ad libitum. Prior to treatment, mice were acclimated to laboratory conditions for two weeks. During this time, the mice were examined and found to be free from lesions of murine pneumonia complex and of oropharyngeal *Pseudomonas* sp.

BrdCyd labeling. BrdCyd labeling has been described elsewhere [14, 21]. Briefly, BrdCyd labeling was accomplished with indwelling osmotic minipumps filled with 150 m*M* BrdCyd (Calbiochem-Behring, San Diego, CA) in sterile, pyrogen-free water. Minipumps (Alza Corporation, Palo Alto, CA) were implanted subcutaneously on the dorsal surface during chloralhydrate anesthetization.

313-nm-light irradiation. Murine bone marrow cells, suspended at 5.0×10^6 cell/ml in Dulbecco phosphate-buffered saline, were irradiated with monochromatic 313-nm light (10-nm band width) at a flux of 15.0 $J/m^2/s$. The irradiation source consisted of a 1 kW Hg-Xe lamp (Oriel Corporation, Stamford, CT) filtered through 80 mm of 1.0×10^{-4} *M* dAde and focused onto a monochromator (model 7024, Oriel Corporation). The flux was determined with a model 8334A thermopile and microammeter (Hewlett-Packard, Palo Alto, CA).

GM-CFC assay. On each day of the infusion period, cell suspensions were prepared of pooled axially derived bone marrows from the femurs of three mice. GM-CFC [16] were assayed by the double-layer agar technique. The agar medium was plated into 60-mm plastic Petri dishes as follows: bottom layer, 2 ml of 1.0% agar (Bactoagar, Difco, Detroit, MI), 2 ml double-strength medium, 100 μl pregnant-mouse-uterus extract [17], 150 μl mouse L-cell-conditioned medium [18]; top layer, 1 ml of 0.66% agar and 1 ml double-strength medium. Double-strength medium consisted of Connaught Medical Research Laboratory (CMRL, Toronto, Canada) 1066 medium containing 10% (vol/vol) fetal calf serum, 5% (vol/vol) horse serum, 5% (wt/vol) trypticase soy broth, 20 g/ml L-asparagine, and antibiotics. After 10 days of incubation at 37°C in 5% humidified CO_2 in air, GM-CFC were scored as colonies of more than 50 cells. For HPP-CFC, the same double-layer agar technique was employed as by Metcalf and Johnson [19], but rat spleen cell-conditioned medium (CM) and PMUE were used as colony stimulants. Single-cell suspensions of rat spleen were prepared at a concentration of 16×10^6 cells/ml of medium. The pokeweed-mitogen–spleen-cell CM was prepared as described by Metcalf and Johnson [19]. Rat spleen CM and PMUE were each used at 6% (vol/vol). The culture medium contained 25% heat-inactivated, pooled human serum. The marrow cells were plated at 5×10^4 cells/dish. An eyepiece graticule was used to assess colony size. All colonies greater than 2 mm in diameter were considered to be derived from HPP-CFCs. Experiments were routinely performed in triplicate with the mean and standard errors reported on the Figures. The data in Figure 5 represent duplicate experiments.

Results

The time dependence of the BrdCyd labeling of GM-CFC was measured for infusion periods of 1–8 days. For these measurements, cell suspensions were exposed to graded fluences of 313-nm light, after which the surviving fraction of GM-CFC was determined. For each of the resulting survival curves, two components of UV sensitivity were detectable. As can be seen in Figure 1, there are two components of cell survival, the more resistant observed for fluences greater than 200 J/m^2. The proportion of cells more resistant to 313-nm light decreased from approximately 70% after one day of infusion to approximately 15% after eight days.

This decrease in the size of the resistant populations was further analyzed by plotting in Figure 2 the proportion of 313-nm-light-resistant cells versus time. GM-CFC survival values at a fluence of ~300

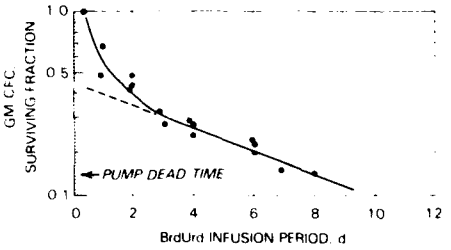

Fig. 2. Decrease in the proportion of GM-CFC resistant to 313-nm light (see text). The fraction of resistant GM-CFC is estimated from the fraction of GM-CFC surviving a fluence of 300 J/m $^{-2}$. *Pump dead time* represents the time from the implantation of minipumps to the time at which BrdCyd is present in the peripheral blood.

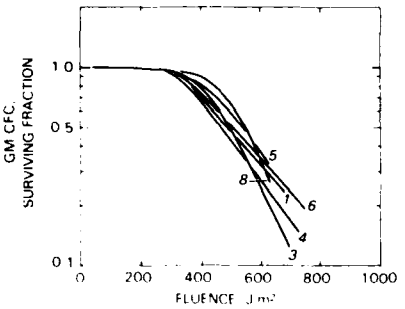

Fig. 3. Survival of the UV-resistant component of the GM-CFC population. The data presented in Figure 1 were normalized to unity at a fluence value of 300 J/m $^{-2}$. The resultant survival curves are indicated by a number corresponding to the number of days of BrdCyd infusion.

J/m $^{-2}$ were used to estimate the proportion of colony-forming cells in the resistant cell population. From the resulting curve, one notes that initially none of the GM-CFC are sensitized to 313-nm light. Over the initial two days of BrdCyd labeling, however, approximately 55% of the GM-CFC rapidly became sensitive to 313-nm light while the remaining 45% were sensitized considerably more slowly. The slowly labeled component demonstrated a half-time for labeling of 5.5 days. In addition to the decrease in the 313-nm-light-resistant cell population, two other characteristics of the survival data in Figure 1 are of interest. First, for fluence values less than 100 J/m $^{-2}$, a shoulder appears on the day-1 and day-2 survival curves. This shoulder was reduced to less than 50 J/m $^{-2}$ for the survival curve determined at day 4 and eliminated by day 8. Secondly, the fluence value of the inflection in the survival curve appears not to be a function of the infusion period. This inflection identifies the 313-nm-light-resistant cell population described

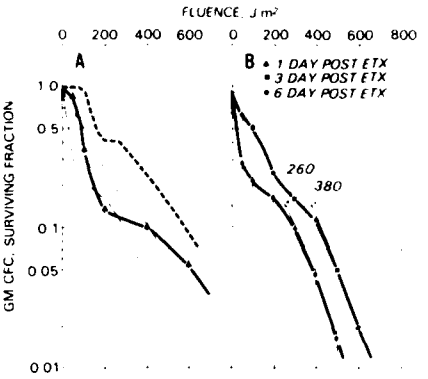

Fig. 4. Effect of endotoxin (ETX) on GM-CFC survival after BrdCyd/313-nm-light treatment. Endotoxin was administered (see *Materials and methods*) 24 h after the implantation of minipumps. For comparison, the survival data (shown by the *dashed line* in A) represent normal GM-CFC after 48 h of BrdCyd infusion.

above. By normalizing to unity the survival values at 300 J/m $^{-2}$ (i.e., fluence value of the secondary shoulder), one sees in Figure 3 that no significant change in the fluence value of this shoulder region occurs during the eight days of BrdCyd infusion. The significance of these shoulder regions will be addressed later in detail.

To determine whether either GM-CFGC population could respond to a proliferative stimulus, endotoxin was administered to a group of mice 24 h after the implantation of BrdCyd-containing minipumps. The intraperitoneal administration of 2.5 μg/mouse of *Salmonella typhosa* endotoxin produced changes in two of the survival curve characteristics described above. The data in Figure 4A show that the fraction of 313-nm-light-resistant cells dropped to approximately 15% 24 h after the endotoxin challenge and was essentially unchanged over the subsequent five days of infusion. In addition, the sensitivity of this population to 313-nm light appeared to increase with time; that is, a comparison of the width of the shoulder at the 15% survival level revealed a measurable decrease as the period of infusion increased. This shift in the shoulder value contrasts directly with the data for the nonchallenged hosts, viz., Figure 3.

Both the G_0 nature and the responsiveness to endotoxin of the GM-CFC precursor cell are characteristics associated with the HPP-CFC [13, 20]. It was decided, therefore, to measure the kinetics of BrdCyd labeling of this cell type. The HPP-CFC was cultured and identified with the techniques detailed in *Materials and methods*. Additional confirmation of the HPP-CFC was provided through the small

Table 1. Decrease in femoral content[a] of HPP-CFC and GM-CFC 2 h after injection with hydroxyurea (Hu)[b]

	HPP-CFC			GM-CFC		
	Pre-Hu	Post-Hu	% Decrease	Pre-Hu	Post-Hu	% Decrease
1)	6471	5805	10.3	18,531	13,496	27.2
2)	8308	8088	2.6	20,689	11,844	42.8
3)	5420	4607	15.0	18,432	11,300	38.4
4)	7175	6583	8.3	36,600	17,460	52.3
	Mean 9.1 ± 2.6			Mean 40.2 ± 5.2		

[a] Mean values from four individual replicate experiments are shown. Four mice were used per replicate. Femoral contents were pooled. HPP-CFC and GM-CFC assays were performed on the same marrow samples.

[b] Hu, 900 mg/kg, was injected intraperitoneally.

fraction of these cells sensitive to hydroxyurea, a characteristic of the HPP-CFC [13]. The data, shown in Table 1, relate this property and agree well with the published difference between GM-CFC and HPP-CFC [13]. If, indeed, the HPP-CFC form this pool of cells that are precursors of the GM-CFC, then they should survive the BrdCyd/313-nm-light treatment in a predictable manner. They should be noncycling. Therefore, there should be no S-phase population to give rise to a shoulder at 200–300 J/m^2. Further, a plateau in survival should be present for each infusion period. This plateau value should decrease at the rate shown in Figure 2. Survival curves illustrating this ideal behavior and actual HPP-CFC survival data are presented in Figure 5. In Figure 5A, survival curves with no shoulder and a minimum or plateau are shown for increasing periods of BrdCyd labeling. When these plateau values are plotted against their associated infusion periods, the resulting simple exponential curve mimics the curves shown in Figure 2. The survival curves for the HPP-CFC shown in Figure 5B were derived from the mean values of duplicate experiments. Also shown for comparison are the survival curves for the GM-CFC population measured from the same pool of cells as one of the HPP-CFC determinations.

Discussion

The present work reports the cell survival response after BrdCyd 313-nm-light treatment of GM-CFC derived from murine bone marrow. BrdCyd infusion was used to effect BrdUrd labeling via the in vivo deamination of BrdCyd [21, 22]. The kinetics for the BrdCyd labeling have been determined by measuring the time rate of change in sensitivity to 313-nm light of the GM-CFC population.

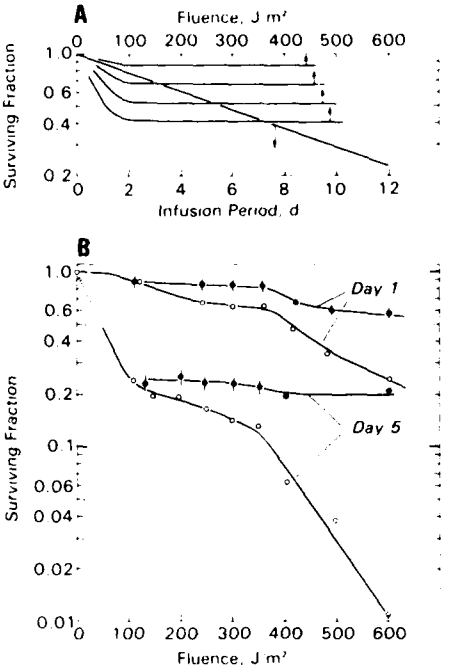

Fig. 5. Survival of HPP-CFC after BrdCyd/313-nm-light treatment. (**A**) Theoretical survival curves for the precursor of the GM-CFC. Minimum (plateau) values in survival were determined from the data in Figure 2. (**B**) Actual survival data for the HPP-CFC (●) and GM-CFC (○) for the infusion periods shown. HPP-CFC data points represent the mean value ± 1 SE for duplicate experiments ($n = 6$). GM-CFC data points are mean values from duplicate samples.

From the data in Figures 1–4, four characteristics of the BrdCyd labeling can be defined. First, as expected, the fraction of the GM-CFC initially resistant to 313-nm light is near unity. During the BrdCyd-labeling period, however, this UV-resistant fraction decreases. The pattern of this decrease in survival discloses two subpopulations with significantly differing labeling kinetics. The more rapidly labeled GM-CFC subpopulation comprises approximately 55% of the total GM-CFC and the more slowly labeled cells comprise the remaining 45%. Secondly, the sensitivity to 313-nm light of the UV-resistant subpopulation does not change with time. However, a steady-state decrease is seen in the proportion of cells resistant to 313-nm light. This decrease is approximately exponential with a half-time of 5.5 days. Third, the proportion of UV-resistant cells decreases markedly in the first 24 h for mice injected with endotoxin. Lastly, in contrast with the normal steady-state response, mice injected with endotoxin exhibit an increase in the sensitivity of the UV-resistant GM-CFC component; this effect

is observed by a decreasing shoulder value with increasing periods of BrdCyd infusion.

To interpret these results, it is first necessary to consider the possible routes for the in vivo BrdCyd labeling of any population of proliferating cells. From data obtained in vitro [22], one knows to expect two components of BrdCyd labeling, both with rapid kinetics (i.e., half-times of less than one cell cycle). These two components arise through (a) the incorporation by cells in the S-phase of a sufficient amount of BrdUrd to be conditionally lethal upon exposure to UV light, and (b) the progress into S-phase of cells initially not synthesizing DNA. However, in vivo a third route (viz., the differentiation into the compartment being assayed of cells labeled in the precursor cell compartment) represents an alternate pathway for the accumulation of labeled cells. This pathway for the accumulation of BrdUrd-labeled cells appears to be apropos for the UV-resistant GM-CFC. Unlike the data for experiments performed in vitro, which show near-maximum sensitivity to 313-nm light after one or two generation times, the data presented here show a population of GM-CFC that is fully sensitized only after some eight or more days, a period much longer than the GM-CFC generation time that would be estimated from the results of suicide experiments, viz., approximately 12 h [23]. Moreover, the UV sensitivity of this population does not change with time, yet it has been shown earlier that these cells have an S-phase fraction of approximately 50% [14]. Thus, these cells are not themselves postmitotic. Since these GM-CFC are cycling in the steady state but are "seeing" the BrdCyd label for the first time, it is likely that they have recently entered the assayable GM-CFC compartment from a noncycling, or G_0, population not assayable under the same conditions.

If the GM-CFC that are initially resistant to 313-nm light are supplied to the GM-CFC compartment directly from a pool of proliferating cells, their UV sensitivity would presumably increase with time. Thus, in time, the fluence value for the shoulder region of the survival curve would decrease and the slope of the survival curve would increase. This does not happen. If the precursor population were composed largely of a G_0 fraction, however, those GM-CFC derived from the G_0 would possess just such properties as described for the slowly labeled 313-nm-light-resistant cells; that is, the shoulder value would not change with time, but the proportion of resistant cells would. Furthermore, the rate of the decrease with time of the proportion of these cells resistant to 313-nm light implies that the precursor population is itself supplied at a rate consistent with

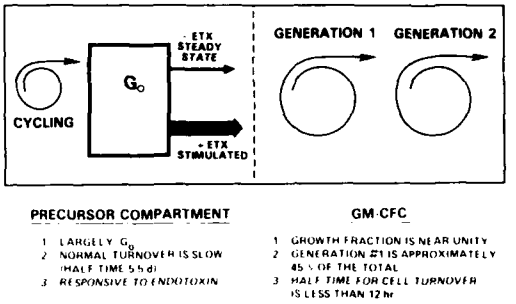

Fig. 6. Kinetics model for GM-CFC production (see text for explanation).

a 5.5-day half-time for the turnover of the G_0 cell pool. Furthermore, the G_0 nature of the precursor of the GM-CFC requires the precursor population to be either fully labeled or completely unlabeled with BrdCyd. This point will be important later.

The GM-CFC precursor pool was also manipulated through its response to a stimulus to proliferate. After the administration of endotoxin, this G_0 pool was exhausted. Whether this occurred through cell proliferation or cell mobilization from the marrow cannot be determined from these data alone. The effect of each would be the same; viz., each process would remove unlabeled GM-CFC from the population responsible for the time-dependent increase in sensitivity to 313-nm light. Interestingly, after the administration of endotoxin and the subsequent depletion of the G_0 pool, a time-dependent increase in sensitivity to 313-nm light occurred (Fig. 4). This effect was most likely due to a recent episode of DNA synthesis for the cells filling this GM-CFC precursor pool. This increased sensitization to UV light may also occur in the absence of an endotoxoin challenge and simply have been obscured in the unchallenged animal by the presence of the large fraction of precursor cells that are normally in a G_0-phase and thus are not labeled by the BrdCyd. Therefore, this increase in sensitivity to UV light apparently induced by the endotoxin may not be dependent upon the endotoxin challenge but may only have been rendered detectable by it.

A model that is consistent with these findings is presented in Figure 6. Although the model is quite general, it is constrained at several points by the present data. For example, the model has been limited to two generations of GM-CFC by the high percentage of GM-CFC that appear to be in the first generation of labeling (i.e., 45% shown in Fig. 2). Furthermore, the G_0 pool is shown as a precursor pool and not a GM-CFC reservoir. This is due prin-

cipally to the high S-phase content of both GM-CFC moieties [14]. The previously reported lower buoyant density of the GM-CFC that are being labeled for the first time is a further indication that first-generation GM-CFC are progeny of the physiologically distinct precursor pool [2, 3, 14]. The final constraint upon the model is that the GM-CFC compartment is being fed by a separate cell type and not by self-renewal. This property has been suggested by others on the basis of the inability to propagate GM-CFC in vitro [24, 25]. Here, the evidence against self-renewal is that first-generation GM-CFC are labeled much more slowly than the rate at which the GM-CFC are cycling. Self-renewal in the GM-CFC compartment thus appears to be limited to, at most, two generations.

The cell kinetics of one candidate for the GM-CFC precursor, the HPP-CFC [9, 13], were measured, and the data are presented in Figure 5. This cell type has previously been shown to be both slowly proliferating, if at all, and responsive to endotoxin challenge [20]. Both of these are properties consistent with the data presented here for the GM-CFC precursor. Further, from the present data, both qualitative and quantitative predictions can be made concerning the survival after 313-nm-light irradiation of the HPP-CFC. If the HPP-CFC constitute the pool of cells in G_0 that feed into the GM-CFC, the HPP-CFC should be composed initially of cells largely not capable of being labeled during the first 24 h of BrdCyd infusion. Secondly, since a G_0 population by definition has no cells in the S phase, there should be no cell population to produce a shoulder on the UV survival curve [26, 27]. Therefore, the survival curves for the HPP-CFC should possess two components: one fully sensitive and the other fully resistant to the BrdCyd/313-nm-light treatment. Further, the proportion of fully resistant HPP-CFC should decrease in a manner quantitatively similar to the decrease in the 313-nm-light-resistant GM-CFC shown in Figure 2.

The data in Figure 5 show that the HPP-CFC labeled for one day are largely not sensitized to 313-nm light. However, a minor population of cells gives rise to a shoulder on the survival curve. For day 5, no such shouldered population was observed. Quantitatively, the population of cells resistant to 313-nm light is below the values predicted from the data presented in Figure 2. For both day 1 and day 5 of the BrdCyd infusion, the nonsensitized fraction is approximately 30% below the predicted values. Since 30% is also the approximate proportion of cells giving rise to the shoulder on the survival curve for day 1 of the infusion, this population may simply be a GM-CFC contaminant of the HPP-CFC assay. In fact, such a contaminant has been reported by others when human serum and mouse spleen-conditioned medium are used to induce the expression of HPP-CFC [13]. Using the plateau survival values from Figure 5, the half-time for turnover of the HPP-CFC is approximately four days. These quantitative differences notwithstanding, the qualitative pattern of survival of the HPP-CFC after BrdCyd/313-nm-light treatment is consistent with the predictions made for the GM-CFC precursor. Although these data support the notion that the HPP-CFC give rise to the GM-CFC, there is no indication from these assays of the nature of the proliferative cell population that supplies the HPP-CFC. The present data suggest that, if the HPP-CFC is the precursor of the GM-CFC, then the HPP-CFC must be supplied from a proliferative cell compartment at a rate that, in the steady state, is consistent with a 5.5-day half-time for cell turnover.

Acknowledgment

This work has been supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00052.

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Exp. Hematol. 13:847–854 (1985)

Experimental Hematology

Rat Colony-forming Unit Spleen Is OX7 Positive, W3/13 Positive, OX1 Positive, and OX22 Negative

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(Received 29 October 1984; in revised form 19 March 1985, accepted 29 March 1985)

Abstract. Using the monoclonal antibodies OX7 HL, W3/13 HLK, OX1 HLK, OX22, and the technique of fluorescence activated cell sorting, it was possible to characterize the phenotype of the rat marrow CFU-S as OX7 upper 20% positive, W3/13 lower 50% positive, OX1 positive, and OX22 negative. OX7 recognizes an antigenic determinant expressed on the Thy1 glycoprotein, W3/13 recognizes a determinant expressed on some sialoglycoproteins, OX1 recognizes all four apparent molecular weight forms of leukocyte common antigen, while OX22 recognizes only the high molecular weight forms of leukocyte common antigen. It was determined that the concentration of OX7 upper 20% positive, W3/13 lower 50% positive, OX1 positive, and OX22 negative cells in the marrow was 3085 ± 1446/10^6 cells. For comparison, the calculated concentration of marrow stem cells using a 2-h seeding efficiency was found to be 501 CFU-S/10^6 cells and, using a 24-h seeding efficiency, it was found to be 1415 CFU-S 10^6 cells. Although requiring further refinements, these results suggest that an assessment of CFU-S marrow concentration might be achieved using multiparameter flow cytometry. Also, a technique for the conjugation of the Fab' fragment of the monoclonal antibody OX7 to phycoerythrin is described.

Key words: Stem cell – OX7 – W3/13 – OX1 – OX22 – Rat CFU-S

There are three major glycoproteins found on rat thymocytes that account for approximately 50% of the total membrane glycoprotein and nearly all of its sialic acid [1]. These glycoproteins are Thy1, leukocyte sialoglycoprotein (LSGP), and leukocyte common antigen (L-CA). There is both direct and indirect evidence that these three glycoproteins are also found on the rat colony-forming unit spleen (CFU-S).

Thy1 antigen was first described in the mouse where it was shown to be present on T cells, thymocytes, and the brain [2]. Thy1 is also found on thymocytes and neural tissues of rats, but not on peripheral T cells [3, 4]. In the rat, expression of Thy1 appears earlier in the development of hematopoietic and lymphoid cells than in the mouse. Unlike the murine CFU-S, which expresses extremely low levels of Thy1 [5], the rat hematopoietic stem cell [6] and T- and B-lymphocyte stem cells [7, 8] express high levels of Thy1. This fact has enabled Goldschneider et al. [9] and Castagnola et al. [10] to purify substantially the rat CFU-S using the technique of fluorescence activated cell sorting. In the rat, Thy1 is recognized by the monoclonal antibody OX7 [11].

The monoclonal antibody W3/13 labels a rat LSGP found on thymocytes, T cells, and the brain, but not on B cells [12]. In this respect, the tissue distribution of the W3/13 antigen is similar to Thy1. In the marrow, however, W3/13 is found on granulocytes plus some other nucleated cells while OX7 labels cells with a lymphoid morphology [13]. Recently, Dyer and Hunt [14] have demonstrated that W3/13 positive bone marrow cells are capable of permanently repopulating the T- and B-cell compartments of an irradiated host. Because both lymphocytes and granulocytes are believed to be derived from a common pluripotent hematopoietic stem cell (CFU-S), it is likely that the expression of W3/13 antigen extends back into the CFU-S compartment.

L-CA has been detected on over 95% of rat bone marrow cells [15], making it likely that L-CA will be found on rat CFU-S. Analysis of rat L-CA by electrophoresis in sodium dodecyl sulfate gels indicates that rat L-CA exists in four forms of 222,000, 200,000, 190,000, and 180,000 apparent molecular weight. The monoclonal antibody OX1 recognizes all four apparent molecular weight forms while the monoclonal antibody OX22 recognizes only the high molecular weight forms [16]. It has been reported

Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

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that the high molecular weight forms of murine L-CA are not expressed on the murine CFU-S [17] and therefore the possibility exists that the rat CFU-S also does does not express the high molecular forms of rat L-CA.

From the work cited above, it is hypothesized that the phenotype of rat CFU-S is OX7 positive, W3/13 positive, OX1 positive, and OX22 negative. The purpose of this study was to develop the appropriate immunofluorescent reagents and labeling protocols enabling this hypothesis to be tested using the technique of fluorescence activated cell sorting.

Materials and methods

Rats. Lewis rats were obtained from Charles River, MA, at four weeks of age. Experiments were performed at eight weeks of age.

Cells. Rats were killed by ether anesthesia. Single-cell suspensions from various lymphoid organs were prepared in phosphate-buffered saline containing 10% fetal calf serum (PBS–10% FCS). Red blood cells were lysed using an ammonium-chloride–potassium buffer.

CFU-S assay. A total of 1×10^3 to 1.5×10^6 nucleated rat bone marrow cells in 1 ml PBS–2% FCS was injected into irradiated rat recipients (900 rad total body radiation, 40 rad min ^{60}Co). The spleens were removed 11–12 days after injection [18] and fixed in Bouin solution.

Antibodies. OX7 HL ascites fluid, FITC–W3 13 HLK, and FITC–OX1 HLK were purchased from Pel Freeze Biologicals (Rogers, AR). OX22 HL ascites fluid was purchased from Accurate Chemical (Westbury, NY). FITC–F(ab')$_2$ goat-antimouse IgG (Fc specific) was purchased from Cappel Labs (West Chester, PA).

Conjugation reagents. Phycoerythrin R (PE), its pyridyldisulfide derivate, dithiothreitol (DTT), and succinimidyl-3-maleimidyl benzoate (SMB) were purchased from Molecular Probes (Junction City, OR).

Conjugation of OX7 to PE. A total of 0.1 ml OX7 ascites was diluted to 0.3 ml with 0.2 *M* $NaPO_4$ 0.1 *M* NaCl pH 7.0; 15 lambda of SMB solution (4.33 mg SMB dissolved in 0.1 ml in *N,N*-dimethyl formamide) was added and the reaction stopped 1 h later by passage over a P-10 gel filtration column equilibrated with 0.1 *M* $NaPo_4$ 0.1 *M* NaCl pH 7.5. The solution was immediately mixed with PE–SH (which had been previously generated from the PE-S-S-pyridyl derivative by reduction with 50 m*M* DTT [19]) at a final wt wt concentration of 1 mg PE 1 mg OX7. After 24 h, the PE–OX7 conjugate was separated from the nonconjugated material by passage over a TSK–G3000SW column (LKB, Gaithersburg, MD).

Preparation of PE–OX7Fab'. Immunoglobulin was prepared from OX7 ascites fluid by precipitation with 16% (wt wt) $NaSO_4$. The IgG was degraded with 2% (wt wt) pepsin in 0.1 *M* Na acetate pH 4 for 20 h at 37°C [11]. Fab' fragments were generated from the F(ab')$_2$ preparation by reduction with 20 m*M* DTT in 0.1 *M* NaCl–0.1 *M* borate–0.05 *M* citrate–2 m*M* EDTA pH 5.0 at 24°C for 90 min. The Fab'–SH fragments were separated from the DTT by passage over a P-10 column equilibrated with 0.1 *M* $NaPO_4$/0.1 *M* NaCl pH 7.5 and allowed to react with maleimide–PE. The maleimide–PE was prepared as described above for maleimide–OX7. After 24 h, the PE–OX7Fab' conjugate was separated from the remaining material by passage through a TSK–G3000SW column.

Cell labeling. Bone marrow cells were incubated with the conjugated antibody(ies) at 4°C for 30–45 min. For unconjugated antibodies, bone marrow cells were incubated with the primary (monoclonal) antibody at 4°C for 30–45 min, washed twice (4 min centrifugation at 500 *g*), resuspended in PBS–10% FCS plus the FITC-labeled secondary antibody, and incubated at 4°C for 30–45 min. Labeled cells were washed twice (4 min centrifugation at 500 *g*) and resuspended in PBS–2% FCS, at a concentration of 5–0.5 $\times 10^6$ cells/ml for cell analysis and sorting on the FACS-II. All reagents were titered to insure maximum labeling.

Cell sorting. Dual parameter flow cytometry measurements and sorting were made with the FACS-II (Becton-Dickinson FACS Systems, Mountain View, CA). The argon laser was tuned at 488 nm and run at 400 mW. The emitted fluorescence light was passed through a 520-nm long-pass filter and 530-nm series-D color-glass filter (Ditric Optics, Hudson, MA) and through a dichroic (570 nm) beam splitter (Becton-Dickinson, Mountain View, CA) to two photomultiplier tubes. The green photomultiplier tube was shielded with a 530-nm band-pass filter and a 540-nm short-pass filter (Ditric Optics). The red photomultiplier was shielded with a 580-nm series-D color-glass filter and a 590-nm band-pass filter (Ditric Optics). The green PMT was set at 850 V and the red PMT at 650 V. The compensation for the red fluorescence channel was set at 3.6. No compensation was needed for the green fluorescence channel.

In the results reported for the cell-sorting experiments, all positive sorts were greater than 92% pure while all negative sorts were greater than 95% pure as determined by running the sorted cells back through the FACS-II.

CFU-S seeding efficiency. The 2-h and 24-h seeding efficiencies *f* for CFU-S were determined according to the method of Siminovitch et al. [20]. Briefly, 1.06×10^8 marrow cells from a pool of five donor rats were injected into three intermediate recipients that had previously been irradiated (900 rad at 40 rad min ^{60}Co) either 2 h or 24 h earlier; 2 h later the intermediate recipients were killed and a splenic cell suspension was prepared. One-tenth of an equivalent spleen was injected intravenously (i.v.) into ten secondary-irradiated (900 rad at 40 rad min ^{60}Co) recipients; 11 or 12 days later the number of spleen colonies was scored. The CFU-S content of the original cell suspension was determined as described above.

Results

Cytotoxicity of OX7, W3 13, OX1, and OX22 monoclonal antibodies

The first experiment listed in Table 1 shows that labeling marrow cells with PE–OX7 and FITC–W3 13 resulted in a decrease in the number of CFU-S that could be detected in the spleen 11 or 12 days after injection. Subsequent experiments (Table 1) indicated that this could be attributed solely to the

Table 1. Effect of monoclonal antibodies on the development of rat bone marrow CFU-S

Exp	Antibody	CFU-S 10^6 cells (mean ± SE) Day 11[a]	Day 12
1	None	29.8 ± 4.3 (6)[b]	37.6 ± 4.6 (5)[b]
	FITC-W3/13 + PE-OX7[c]	6.0 ± 1.6 (6)	6.7 (2)
2	None	30.8 ± 5.2 (4)	32.5 ± 3.9 (4)
	FITC-W3/13	37.0 ± 3.0 (4)	35.5 ± 6.5 (2)
3	None	20.4 ± 1.4 (5)	32.6 ± 1.7 (5)
	PE-OX7	9.4 ± 2.5 (5)	9.5 ± 3.2 (4)
	FITC-OX7	2.5 ± 0.7 (6)	3.3 ± 2.0 (3)
	OX7[d]	2.8 ± 0.9 (6)	3.3 ± 0.3 (4)
4	None	34.0 ± 8.0 (2)	37.0 ± 1.0 (2)
	PE-OX7Fab	30.0 ± 8.2 (3)	–
	PE-OX7Fab' + FITC-W3/13	32.2 ± 2.5 (5)	–
5	None	33.0 ± 1.7 (4)	36.7 ± 3.5 (3)
	OX1	7.9 ± 4.7 (3)	7.9 ± 0.8 (3)
	OX22	22.1 ± 3.2 (3)	34.2 ± 0.3 (3)
6	None	32.0 ± 7.8 (5)	49.9 ± 10.6 (5)
	OX1	4.9 ± 1.4 (6)	12.3 ± 2.7 (4)
	FITC-OX1	15.3 ± 2.5 (5)	10.8 ± 2.0 (5)

[a] The day at which spleens from recipients were removed for examination.
[b] Values in parentheses are number of spleens examined.
[c] All antibody-fluorochrome preparations were titrated to give maximum fluroescence as analyzed by flow cytometry.
[d] OX7 and OX1 were used at a final dilution of 4 lambdas of 1:100 dilution of ascites fluid per 10^6 cells in 0.1 ml.

OX7 monoclonal antibody and could be avoided by using the PE-OX7 conjugate in its Fab' form. Other experiments listed in Table 1 show that labeling marrow cells with OX1 and FITC-OX1 also resulted in a decrease in the number of CFU-S that could be detected in the spleen 11 or 12 days after irradiation. This loss or cytotoxic killing of CFU-S was not observed when bone marrow cells were incubated with OX22 or W3/13 monoclonal antibodies.

Single parameter sorting of OX7, W3/13, OX22, and OX1 labeled marrow cells

Figure 1 shows that labeling nucleated bone marrow cells with FITC-W3/13 defined two cell populations: a bright population and a dim-negative population. Sorting the cells according to the windows a, b, c, and d as identified in Figure 1 demonstrated that rat CFU-S are W3/13 positive (Table 2). Further, CFU-S appeared in the lower 50% of the W3/13 bright or positive fraction defined as window c (Table 2, exp. 2). It should also be noted that labeling

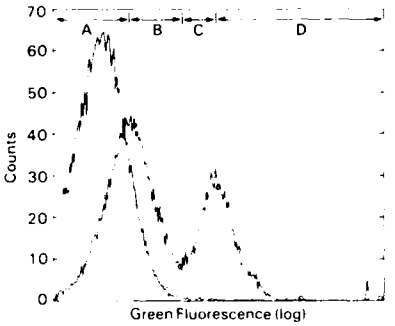

Fig. 1. Relative fluorescence distribution of marrow cells incubated with FITC-W3/13. The *letters* refer to the sort windows. The distribution is from a total count of 30,000 cells. Unlabeled cells ——; FITC-W3/13-labeled cells –o–o–. Mean % positive cells from ten determinations was 39.7 ± 2.8 SE.

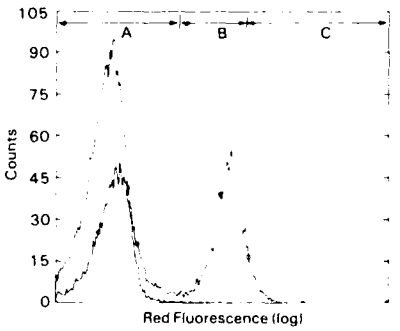

Fig. 2. Relative fluorescence distribution of marrow cells incubated with PE-OX7Fab'. The *letters* refer to the sort windows. The distribution is from a total count of 30,000 cells. Unlabeled cells ——; PE-OX7Fab'-labeled cells –o–o–. Mean % positive cells from ten determinations was 52.6 ± 2.6 SE.

cells with PE-OX7Fab' did not displace FITC-W3/13 from CFU-S (Table 2, exp. 3).

Figure 2 shows that 51% of all nucleated bone marrow cells could be labeled with PE-OX7Fab'. Similar results were obtained with FITC-OX7 and PE-OX7 (data not shown). The CFU-S was found primarily in the upper 20% of the OX7 staining cell population, which is window c (Table 2, exp. 4).

Sorting bone marrow cells labeled with FITC-OX1 demonstrated that those CFU-S which escaped the cytotoxicity of FITC-OX1 are OX1 positive (data not shown).

Figure 3 shows that 63% of bone marrow cells could be labeled with OX22 plus FITC-F(ab')$_2$ goat-antimouse IgG (Fc specific) antibody. Sorting these cells according to windows a and b (Table 2, exp. 5) demonstrated that CFU-S are OX22 negative.

Table 2. Development of spleen colonies from single-parameter sorted cells

Exp	Reagent	Sort window[a]	% Cells in window	CFU-S/10[6] cells (mean ± SE) Day 11[b]	Day 12	Maximum calculated enrichment:[f] CFU-S/10[6] Day 11	Day 12
1	FITC-W3/13	a + b	56	–	6.9 ± 1.6 (6)[c]	–	65
		c + d	44	–	53.6 ± 3.9 (7)	–	83
		Irradiated control[d]		–	0.8 ± 0.5 (5)	–	–
2	FITC-W3/13	a	23	0 (1)[e]	–	134	–
		b	28	3.3 ± 1.9 (3)	–	110	–
		c	25	Confluent[e] (4)	–	124	–
		d	25	6.5 ± 1.2 (4)	–	124	–
3	FITC-W3/13 and PE-OX7Fab' but sorted only on FITC-W3/13 green fluorescence	a + b	54	6.3 ± 2.1 (4)	–	57	–
		c + d	46	25.0 ± 4.5 (4)	–	67	–
		Irradiated control		2.5 ± 0.5 (2)	–	–	–
4	FITC-W3/13 and PE-OX7Fab' but sorted only on PE-OX7Fab' red fluorescence	b	42	3.0 ± 0.9 (4)	3.1 ± 1.1 (3)	74	87
		c	10	304.0 ± 25.2 (5)	328.4 ± 40.3 (5)	309	365
		Irradiated control		0 (1)	3.0 (1)	–	–
5	OX22 + FITC-F(ab')$_2$ GAMIgG(Fc)	a	36	95.5 ± 18.1 (4)	113.4 ± 11.9 (4)	86	101
		b	64	6.2 ± 2.2 (4)	7.4 ± 2.5 (2)	48	57

[a] See Figure 1, Figure 2, or Figure 3.
[b] Day at which spleens from recipient rats were removed for examination.
[c] Number in parentheses refers to number of spleens examined.
[d] Expressed as number of nodules per spleen.
[e] 0.50 × 10^6 cells injected results in confluence of colonies in recipient spleen.
[f] See footnote to Table 3.

Dual parameter sorting of OX7 and W3/13 or OX7 and OX22 double-labeled cells

To determine whether both PE-OX7 and FITC-W3/13 could simultaneously identify CFU-S as would be indicated by experiments 3 and 4 in Table 2, a double-labeling and sorting experiment was performed. As seen in Figure 4, following incubation with PE-OX7Fab' and FITC-W3/13 and analysis on FACS-II, three major marrow subpopulations were distinguished. In this analysis it was determined that 42% of marrow cells were W3/13 negative and OX7 positive, 14% were double negative, and 39% were W3/13 positive and OX7 negative, while only 4% were double positive. After sorting and injection of the double positive fraction into irradiated recipients, however, it was determined that 82% of all recovered CFU-S were found in the double positive window (Table 3, exp. 1). When marrow cells were sorted for the OX7 upper 20% positive and the W3/13 lower 50% positive, an 88-fold enrichment of CFU-S was realized (Table 3, exp. 2).

By double labeling bone marrow cells with PE-OX7Fab' and OX22 plus FITC-F(ab')$_2$ goat-anti-mouse IgG (Fc specific) (Fig. 5), it was found that 37% of marrow cells were double negative, 6% were OX7 positive and OX22 negative, 50% were double positive, and 7% were OX7 negative and OX22

Table 3. Development of spleen colonies from dual parameter sorted cells

Exp	Sort window[a]	% Cells in window	CFU-S 10^6 cells (mean ± SE) Day 11	Day 12	Maximum calculated enrichment[c] CFU-S 10^6 cells Day 11	Day 12
1	All W3 13 positive OX7 positive	4.00	326.8 ± 20.8 (4)	445.0 ± 57.8 (4)	772	913
	All cells other than W3 13 positive, OX7 positive	96.00	7.5 ± 5.6 (3)	4.0 ± 0.5 (3)	32	38
2	W3 13 fraction c OX7 fraction c	0.45	2540.0 ± 157.9 (4)	3180.0 ± 123.8 (4)	6867	8111
	Irradiated control[b]		0.0 (1)	3.0 (1)		
3	All OX22 negative, OX7 fraction c	0.47	4380.0 ± 506.4 (5)	4600.0 ± 214.5 (6)	6574	7766
	All cells other than OX22 negative, OX7 fraction c	99.53	3.4 ± 0.7 (5)	2.3 ± 0.9 (4)	31	37

[a] See appropriate Figures.

[b] Expressed as nodules per spleen.

[c] Due to the large number of rats required for these experiments, it was not always possible to run a normal rat marrow control with each experiment. Therefore, the maximum calculated enrichment was calculated from an average of eight CFU-S determinations using normal marrow that yielded 30.9 ± 4.8 CFU-S 10^6 cells as measured at day 11 and 36.5 ± 3.1 CFU-S 10^6 cells at day 12. Assuming a 100% recovery of CFU-S and that all CFU-S are defined by the parameters listed in the second column, these normal CFU-S incidence values were then divided by the percent cells in windows for the respective experiments to give a maximum calculated enrichment.

positive. It should be noted that the second antibody also tagged OX22 negative and sIg positive cells. Sorting on the parameters upper 20% OX7 positive and OX22 negative resulted in a 127-fold enrichment of CFU-S (Table 3, exp. 3). It was calculated that 90% of all recovered CFU-S were defined by these parameters.

Concentration of OX7 upper 20% positive, W3 13 lower 50% positive, and OX22 negative cells in the marrow

To arrive at the concentration of cells in the marrow characterized by the phenotype OX7 upper 20% positive, W3 13 lower 50% positive, and OX22 negative, OX22 negative cells were first separated from OX22 positive cells by sorting on the FACS-II and then relabeled with PE–OX7Fab' and FITC–W3 13 and reanalyzed on the FACS-II. Using this approach it was possible to estimate that the number of OX7 upper 20% positive, W3 13 lower 50% positive, and OX22 negative cells in the marrow is 3085 ± 1446 10^6 cells (Table 4).

Estimation of the CFU-S concentration in rat bone marrow based on the seeding efficiency f

In the mouse and rat, the concentration of CFU-S 10^6 marrow cells can be estimated by use of the

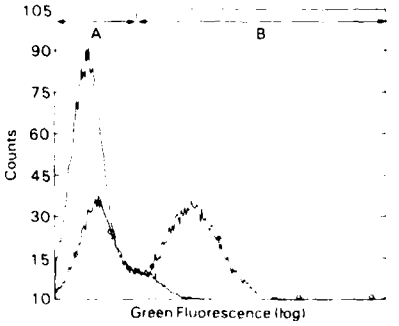

Fig. 3. Relative distribution of marrow cells incubated with OX22 plus FITC–F(ab')$_2$ goat–antimouse IgG (Fc specific). The *letters* refer to the sort windows. The distribution is from a total count of 30,000 cells. Cells labeled with FITC–F(ab')$_2$ goat–antimouse (Fc specific) ——, or OX22 plus FITC–F(ab')$_2$ goat–antimouse (Fc specific) –o–o–. Mean % negative cells from eight determinations was 41.0 ± 2.2 SE.

seeding efficiency *f*. This factor takes into account that only a small percentage of the CFU-S injected i.v. ever lodge in the recipient spleen. Two estimates of *f* that are most widely used are those that are derived from mice or rats irradiated 2 h or 24 h prior to their use as intermediate recipients. The 24-h postirradiation seeding efficiency is believed to take into account postirradiation splenic shrinkage and therefore the 24-h *f* is usually less than the

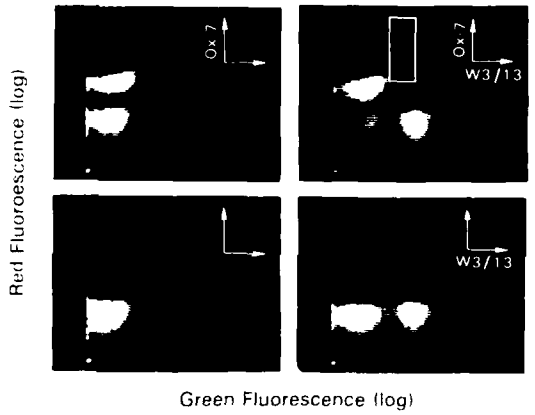

Fig. 4. Two-color fluorescence analysis of marrow cells labeled with PE–OX7Fab' and FITC–W3 13; 30,000 cells were analyzed. The *white rectangle* approximates the sort window defined by the parameters OX7 upper 20% positive and W3 13 lower 50% positive. Mean % distribution ± SE from six determinations: double positive cells 4.3 ± 1.4; OX7 positive, W3 13 negative cells 48.2 ± 4.8; OX7 negative, W3 13 positive cells 32.1 ± 2.7; double negative cells 15.0 ± 3.7.

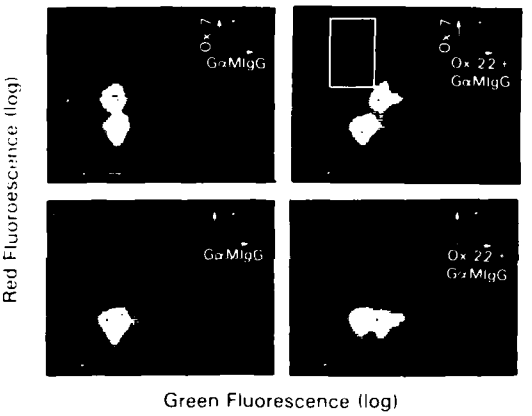

Fig. 5. Two-color fluorescence analysis of marrow cells labeled with PE–OX7Fab' and OX22 plus FITC–Ftab'; goat-antimouse (Fc specific). 30,000 cells were analyzed. The *white rectangle* approximates the sort window defined by the parameters OX7 upper 20% positive and OX22 negative. Mean % distribution ± SE from three determinations: double positive cells 46.9 ± 1.6; OX22 positive, OX7 negative cells 8.1 ± 0.9; OX22 negative, OX7 positive cells 3.5 ± 1.2; double negative cells 41.4 ± 2.4.

Table 4. Concentration of OX7 upper 20% positive, W3 13 lower 50% positive, and OX22 negative cells in rat marrow

Exp	% OX22 *negative*	% OX22 negative cells* that are OX7 upper 20% positive, W3 13 *lower 50% positive*	OX22 negative, OX7 upper 20% positive, W3 13 lower 50% positive	
			%	Per 10^6 cells
1	30.0	0.61	0.18	1830
2	37.7	0.64	0.24	2413
3	43.0	1.70	0.73	7310
4	43.0	0.18	0.08	778
	38.4 ± 3.0	0.78 ± 0.32	0.30 ± 0.14	3085 ± 1446

* The parameters defining this window were determined using normal marrow before making the measurements with the OX22-negative marrow.

2-h *t*. This pattern was also observed in the present experiments (Table 5). Depending on whether the 2-h or 24-h *t* was used in the calculation, it was determined that rat marrow CFU-S concentration is 501 or 1415 10^6 cells, respectively (Table 5).

Discussion

Using the reagents PE–OX7Fab', FITC–W3 13, and OX22 *plus FITC–Ftab'), goat-antimouse* IgG (Fc specific) and the technique of fluorescence activated cell sorting, it was possible to demonstrate that the phenotype of the rat CFU-S is OX7 upper 20% positive, W3 13 lower 50% positive, and OX22 negative. A combination of OX1 cytotoxicity and cell sorting also demonstrated that the rat CFU-S is OX1 positive.

Significant enrichment of CFU-S was achieved by gating on the set of parameters OX7 upper 20% positive, W3 13 lower 50% positive or OX7 upper 20% positive, and OX22 negative. Gating on the former set of sort parameters resulted in an 88-fold purification of CFU-S, while gating on the latter set of sort parameters resulted in 127-fold purification of CFU-S. Of the several experiments designed to achieve maximum purification of CFU-S, the one yielding the highest purity of CFU-S was that using the sort parameters OX7 upper 10% positive and W3 13 lower 50% positive (data not shown). Enrichment of CFU-S approaching 10,000 CFU-S 10^6 cells was achieved, but only 50% of the recovered CFU-S were found to be characterized by this phenotype.

The concentration of cells in rat marrow with the

Table 5. Determination of seeding efficiency *f*

Cells injected	Recipient	CFU-S/spleen	CFU-S/10^6 cells	*f*	Corrected CFU-S 10^6 cells
		Day 11			
0.775×10^6	Primary	21.8 ± 5.2 (5)	28.12 ± 6.7		
106×10^6	2-h intermediate				
	24-h intermediate				
10% of intermediate spleen	2-h secondary	29.0 ± 3.5 (4)		9.7%	289
	24-h secondary	11.5 ± 2.5 (4)		3.9%	721
Irradiated control		0.8 ± 0.2 (6)			
		Day 12			
0.775×10^6	Primary	35.3 ± 2.9 (3)	45.6 ± 3.8		
106×10^6	2-h intermediate				
	24-h intermediate				
10% of intermediate spleen	2-h secondary	41.4 ± 4.5 (5)		9.1%	501
	24-h secondary	14.5 ± 2.5 (2)		3.2%	1415
Irradiated control		0.6 ± 0.3 (3)			

phenotype OX7 upper 20% positive, W3/13 lower 50% positive, and OX22 negative was determined to be 3085 ± 1446/10^6 cells. For the purpose of discussion, if it is assumed that in the marrow only CFU-S are characterized by this phenotype, then the actual concentration of marrow CFU-S would be twofold to ninefold higher than those values for marrow CFU-S concentration determined using the classically defined seeding efficiencies *f*. Depending on whether a 2-h or 24-h *f* was used, it was calculated that stem cells are present in rat marrow at a concentration of 501 or 1415 CFU-S/10^6 cells, respectively. Alternatively, using the extrapolation method described by Van Bekkum [21] to measure *f*, which generates a value of 0.7% and a rat marrow stem cell incidence of 7000 CFU-S/10^6 cells, it might also be concluded that not all rat CFU-S are characterized by the above-mentioned phenotype.

In the mouse it has been determined that the concentration of marrow CFU-S is between 1090 (2-h *f*) and 3585 (24-h *f*)/10^6 cells [22]. Although these values for murine CFU-S concentration are consistent with that suggested by flow cytometry for rat CFU-S concentration, these values for murine CFU-S concentration are not consistent with the values for rat CFU-S concentration determined using *f*. A possible discrepancy that might arise between the CFU-S assay in the rat and mouse is that in both species the hematopoietic colonies that develop in the spleen are approximately the same size yet the rat spleen is 5–10 times larger than the murine spleen. It is likely that those colonies growing in the interior of the rat spleen would have a higher probability of going undetected by gross examination of the recipient spleen than those interior colonies growing in murine spleen.

An alternative explanation would be that the murine spleen supports the growth of spleen colonies that are not only derived from multipotent CFU-S but also committed CFU-S. The latter type of colonies are observed at eight days after transplantation and some of them disappear by day 12 [23]. Further, the murine eight-day CFU-S has a different phenotype than the 12-day CFU-S [24]. In the rat, no eight-day CFU-S colonies are observed. All spleen colonies are scored between 11 and 12 days after implantation [18]. Thus, the murine CFU-S assay may overestimate the actual concentration of marrow hematopoietic stem cells. The work of Boggs et al. [25] presents evidence that this may be true. Using a limiting dilution assay based on the cure of anemic W/W^v mice by +/+ marrow transplantation, they were able to calculate that the concentration of stem cells in the mouse is approximately 100 CFU-S/10^6 cells.

Acknowledgments

Supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00027. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

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CELLULAR IMMUNOLOGY 94, 480–490 (1985)

Thymic Hormones in Radiation-Induced Immunodeficiency

I. Induction of Mature Interleukin 1 Responsive Cell in the Thymus by Thymosin Fraction 5[1]

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Received March 18, 1985; accepted April 23, 1985

The restorative effect of thymosin fraction 5 (TF5) on the thymus of γ-irradiated mice was examined. Four different mouse strains were used in this study since earlier work determined that the degree of response to TF5 is strain dependent. The responsiveness to comitogenic effect of interleukin 1 (IL-1) was used to measure the rate of recovery of immunocompetent cells in the thymus, since only more mature PNA^-, $Lyt\text{-}1^+\text{-}2^-$ medullary cells respond to this monokine. Contrary to several earlier reports that radioresistant cells repopulating the thymus within the first 10 days after irradiation are mature, corticosteroid resistant, immunocompetent cells, the thymic cells from irradiated mice in all strains used had greatly reduced responses to IL-1. Daily intraperitoneal injections of TF5 increased significantly the responses of thymic cells to IL-1 in 10- to 13-weeks-old C57Bl/KsJ, C57Bl/6, C3H/HeJ, and DBA/1 mice. Older mice, 5 months or more in age, of DBA/1 strain did not respond to treatment with TF5. However, C3H/HeJ mice of the same age were highly responsive. In conclusion, (1) cells repopulating the thymus within 12 days after irradiation contain lower than normal fraction of mature IL-1 responsive cells, (2) thymic hormones increase the rate of recovery of immunocompetent cells in the thymus, and (3) the effect of thymic hormones is strain and age dependent.

INTRODUCTION

Clinical and experimental data have firmly established that the thymus gland is of critical importance for the normal development and acquisition of T-cell function. However, although it is recognized that T-cell proliferation and differentiation take place in the thymus, it is by no means clear how these processes are regulated [reviewed in Ref. (1)].

Much work has been presented to show that soluble substances extracted from the thymus, referred to as thymic hormones, affect many parameters of cell-mediated immune responses and, therefore, may be the inducers of thymic immunocompetence (2–7). Some of these hormone preparations stimulate maturation and proliferation of T cells (8–10). Most of these studies have demonstrated these

[1] Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council.

0008-8749/85 $3.00

effects of thymic hormones on T cells from bone marrow or spleen, whereas studies of effects of thymic hormones on thymocytes themselves are few and controversial. Using thymocytes from children undergoing open heart surgery, Ho *et al.* (11) showed that incubation with thymosin fraction 5 (TF5) or α_1, enhances expression of OKT-3 and decreases TdT markers, an indication of maturational changes for human thymocytes. Following *in vitro* incubation with 100 μg/ml of TF5 murine thymocytes exhibited enhanced production of interleukin 2 (IL-2) and colony-stimulating factor (CSF) (12). TF5 acted on mature PNA cells since an enrichment for PNA cells but not PNA' cells resulted in significantly increased responsiveness. Other reports indicate, however, that incubation of the thymus or thymic cells with thymic hormones did not result in changes in cell markers or in acquisition of functions characteristic of mature medullary thymocytes (13, 14).

The negative results in the above experiments may relate to the complex immunomodulatory mode of action of thymic hormones. The effects exerted by TF5, which is a preparation extracted from bovine thymus and consists of 40–50 polypeptides (15), serve to illustrate this point. TF5 was shown to be most effective in a large number of clinical and experimental systems when used in treatment of immunodeficiency states [reviewed in Ref. (16)]. For example, mouse strains susceptible to infections with *Candida albicans* or low responders in the *in vivo* release of lymphokines, migration inhibitory factor (MIF), or Interferon-γ (IFN-γ), become resistant and highly responsive following daily administration of 5 μg of TF5. Both responses of resistant high-responder mice, however, were lowered following similar administration of TF5 (17, 18). Therefore, it appears that reconstitution of immunologically defective function rather than augmentation of normal levels of responses characterizes the action of hormones such as TF5.

Ionizing radiation has a profound suppressive effect on the thymus gland. A dramatic involution in the murine thymus develops following even low doses (150–200 cGy) of γ irradiation. Regeneration, as measured by weight and mitotic index, begins 5–7 days after irradiation (19). The thymuses of radiation-immunocompromised mice may present a convenient model to observe the effect of thymic hormones on the maturation of the cells in the thymus. We have, therefore, examined the restorative effect of treatment with TF5 on the thymic cells from irradiated mice. Previous work has established that the effectiveness of treatment with TF5 varies widely in different strains of mice. C3H/HeJ mice susceptible to infection with *C. albicans* and low responders in the *in vivo* release of MIF and IFN-γ become resistant and release high titers of the two lymphokines into circulation following daily administration of TF5. However, RF/J and DBA/1 strains, that are also susceptible and low responders, were not affected by hormone administration (17, 18). In addition, C57Bl/KsJ and C57Bl/6 mice, that are normally resistant and high responders, become susceptible and low responders to *C. albicans* when compromised by induction of a diabetic condition (20). These two strains also responded to treatment with thymosin with enhanced resistance, lymphokine release, and delayed footpad reaction to *C. albicans*. The above mouse strains were chosen for this study of the effect of administration of TF5 on the rate of recovery of immunocompetent cells in the thymus of irradiated mice.

Thymic involution that begins at puberty is presently not understood. Since it is possible that this process depends on reduced production and/or responsiveness to thymic hormones, mice ranging in age from 10 weeks to 6 months were included

in the experiments. As a measure of thymocyte function, we have chosen to assay changes in responsiveness to IL-1, since previous work established that only more mature PNA^- $Lyt\text{-}1^+\text{-}2^-$ cells respond by proliferation in this assay (21–24).

In this paper we demonstrate that following irradiation with 450 cGy administration of TF5 accelerates the rate of recovery of IL-1 responsive cells in the thymus. This effect is strain, as well as age dependent.

MATERIALS AND METHODS

Mice. Inbred strains of female mice (C57Bl/KsJ, C57Bl/6J, DBA/1J, and C3H/HeJ) were obtained from Jackson Laboratories, Bar Harbor, Maine. The mice were housed in the Veterinary Medicine Department facility at the Armed Forces Radiobiology Research Institute in cages of 10–12 mice with filter lids. Standard lab chow and HCL acidified water (pH 2.4) were given *ad libitum.* All cage cleaning procedures and daily injections were carried out in a microisolator.

Irradiation. Mice were placed in Plexiglas restrainers and given whole-body irradiation at 0.40 Gy/min by bilaterally positioned cobalt-60 elements. The total dose was 4.5 Gy (450 rad).

Thymosin fraction 5. This was obtained through the courtesy of Dr. Allan Goldstein, Department of Biochemistry, The George Washington University School of Medicine, Washington, D.C. The control fraction, kidney fraction 5, was also kindly provided by Dr. Goldstein. Both lyophilized fractions were diluted in pyrogen-free saline (Travenol Labs.) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin to a final concentration of 10 μg/ml. Each mouse received 0.5 ml daily intraperitoneal injection.

Thymic cell suspensions. Three to six mice per experimental group were sacrificed via ether anesthesia on the days postirradiation as noted. Thymuses were removed, cleared of any parathymic lymph nodes, and placed in Hanks' balanced salt solution (HBSS—GIBCO) on ice. Single cell suspensions were prepared by passing the thymuses through a Millipore screen (20-mm diameter) and then a 23-gauge needle and syringe. The cells were washed two times in HBSS (200 g, 10 min, 4°C), and resuspended in complete medium containing RPMI 1640, 10% calf serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-5} *M* 2-mercaptoethanol, and 2 m*M* L-glutamine. Viability for all cell suspensions was found to always be >95%.

IL-1 preparations. Two preparations of IL-1 were used. IL-1 purchased from Genzyme with a sp act of 100 U/ml was used at a final concentration of 5 and 1 U/ml (Lot Nos. 094a, 095a). IL-1 was also prepared in the laboratory according to Gery *et al.* (25). Briefly, resident peritoneal macrophages were lavaged from C57Bl/6 mice. Cell suspensions containing 2×10^6 cells/ml were allowed to adhere for 2 hr to the surface of plastic Costar 2506 multiwell dishes and then after removal of non-adherent cells, were incubated for 24 hr at 37°C in 5% CO_2 with 20 μg/ml of lipopolysaccharide (Difco, Detroit, Mich.) and 60 μg/ml of silica (gift from Dr. Alison D. O'Brien, Department of Microbiology, Uniformed Services University of the Health Sciences) prepared as specified (26). The supernatants were used in dilutions ranging from 1:50 to 1:250. Controls which consisted of cell culture supernatants to which LPS and silica were added at the termination of the culture did not have any stimulatory effect.

IL-1 assays. The assay was performed as previously described (25). Briefly, triplicate cultures for each IL-1 dilution and background control were set up in 96-well flat-bottom microtiter plates (Costar 3596, Cambridge, Mass.). Two cell concentrations were used in each assay, usually 0.1 ml/well of 3×10^7 cells/ml or 1.5×10^7 cells/ml. PHA (Wellcome Burroughs, Greenville, N.C.) was added to the cell suspensions at a final concentration of 1.0 μg/ml. Following 48 hr incubation at 37°C in 5% CO_2, cells were pulsed with 1 μCi [^{3}H]thymidine per well. The cells were harvested 18 hr later (Skatron Cell Harvester, Sterling, Va.) onto glass filters which were then counted in Scintiverse II on a Mark III Scintillation counter to determine thymidine uptake. Statistical analyses were performed using Student's *t* test.

RESULTS

(1) C57Bl/KsJ. Mice of this strain, when immunocompromised by induction of diabetes, became responsive to TF5 in that they developed enhanced resistance to *C. albicans,* increased titers of MIF and IFN-γ, and increased delayed footpad hypersensitivity (20). We performed three series of experiments, each with 10–12 mice per group. Since variations were detected in the baseline responses in each series, the results of the experiments were not combined. Table 1 summarizes one series of these experiments that compares the magnitude of comitogenic effect of IL-1 in thymocytes from TF5-treated and control mice. Mice were 3 months old at the time of irradiation with 450 cGy. The results are expressed as mean counts per minute (cpm) ± standard deviation (SD) of triplicate culture containing 1.5 or 3.0 $\times 10^6$ thymic cells per well incubated with or without 1:100 dilution of IL-1 preparation (equivalent to 5 units per ml of Genzyme IL-1). The responses to two other concentrations of IL-1 used in each assay indicated the linearity of responsiveness to IL-1 (data not shown). It is apparent that normal C57Bl/KsJ mice can be characterized as low responders to IL-1. The recovery of cells/thymus on a given day after irradiation is very similar for the individual experimental groups and agrees with previously published observations on the initial cell recovery in the

TABLE 1

Effect of Thymosin Fraction 5 on Comitogenic Response of Thymocytes from C57Bl/KsJ Mice to IL-1

	D + 6		D + 9		D + 12	
Treatment	IL-1	Control	IL-1	Control	IL-1	Control
Thymosin fraction 5	23743 ± 7678 (1.1×10^7)	527 ± 284	22432 ± 10489 (8.0×10^7)	444 ± 15	14989 ± 1050 (5.3×10^7)	2673 ± 23
Saline	814 ± 281 (1.0×10^7)	267 ± 66	791 ± 136 (8.0×10^7)	283 ± 33	400 ± 75 (5.2×10^7)	448 ± 41
Irradiated only	1174 ± 553 (1.8×10^7)	721 ± 423	293 ± 139 (9.0×10^7)	548 ± 187	229 ± 71	302 ± 19
Normal, non-irradiated			1709 ± 486	779 ± 175	2287 ± 477	309 ± 3

Note. Thymocytes were recovered at days after irradiation and cultured at a cell concentration of 1.5×10^6 cells/well (for D + 6) or 3.0×10^6 cells/well (for D + 9 and D + 12). Results are mean cpm ± SD of triplicate cell cultures with 5 units/ml of IL-1 or of controls. The numbers in parentheses are the number of cells recovered per thymus.

thymus of irradiated mice (19). A striking difference, however, may be observed in the responsiveness of cells to IL-1. The TF5-treated thymocytes responded significantly more than the control and the saline-treated or irradiated groups. In two additional series of experiments 10-week-old mice were used, since 8- to 10-week-old CD-1 mice respond to IL-1 with peak activity (27). Although much higher number of cells/thymus were recovered from the normal, 10-week-old C57Bl/KsJ mice (ranging from 2.3 to 4.0 $\times$ 10^8 cells), the thymocytes in response to 1:100 dilution of IL-1 incorporated only 2–4 $\times$ 10^3 cpm of ^{3}HTdR. Thus, lower levels of response were observed in thymuses with higher cellularity. At Days 6, 9, and 13 after irradiation the TF5-treated mouse thymocytes from C57Bl/KsJ mice responded at 73, 129 $\pm$ 7, and 80 $\pm$ 60% of normal control responses, respectively. The saline or kidney fraction 5-treated control groups had only 19, 41 $\pm$ 37, and 10 $\pm$ 9%, and irradiated mice had only 40, 8 $\pm$ 11, and 13 $\pm$ 8% of normal control responses on the same days. Therefore, despite the reduced effect of the treatment with TF5 in 10-week-old C57Bl/KsJ mice a marked greater response was still obtained from TF5-treated than from saline/kidney fraction 5-treated, or irradiated mice. We conclude, therefore, that treatment with TF5 in comparison with saline or kidney fraction 5, enhances the recovery of IL-1 responsive cells in the thymuses of irradiated mice.

(2) C57Bl/6. Twelve-week-old C57Bl/6 mice were used in three series of experiments. Since in C57Bl/KsJ mice a strong effect of TF5 was apparent already 6 days after irradiation, the present strain also was examined on this day in all three experiments. Days 12, 14, and 16 were included in addition to Day 6. The results of the three series of experiments are combined in Table 2. It can be observed again that the recovery of cells per individual thymus in different experimental groups on a given day does not vary significantly. A decline in the numbers of cells per thymus occurs on Days 14 and 16. Persistent levels of responses to IL-1, however, were

TABLE 2

Effect of Thymosin Fraction 5 on Comitogenic Response of Thymocytes from 12-Weeks-Old C57Bl/6 Mice to IL-1

Treatment	D + 6	D + 12	D + 14	D + 16
Thymosin fraction 5	1515 $\pm$ 446 ($5.2 \pm 2.5 \times 10^7$)	1920 $\pm$ 974 (2.8×10^8)	1965 $\pm$ 720 (2.3×10^8)	3624 $\pm$ 312 (1.75×10^8)
Kidney fraction 5	380 $\pm$ 208 ($7.2 \pm 2.3 \times 10^7$) $P < 0.001$	169 $\pm$ 182 (3.3×10^8) $P < 0.02$	1466 $\pm$ 575 (1.7×10^8) NS	0 (2.0×10^8) $P < 0.005$
Irradiated only	614 $\pm$ 334 ($8.3 \pm 4.0 \times 10^7$) $P < 0.02$	0 (2.7×10^8) $P < 0.02$	782 $\pm$ 658 (1.7×10^8) $P < 0.03$	1192 $\pm$ 548 (1.1×10^8) $P < 0.05$
Normal, non-irradiated	2533 $\pm$ 1427 ($3.61 \pm 1.3 \times 10^8$)			

Note. Results of three separate experiments on Day 6 and additional experiments from three different series on Days 12, 14, and 16. Results are expressed as Δcpm-mean counts of triplicate cultures stimulated with 5 units/ml of IL-1 minus mean counts of triplicate controls, of 3 $\times$ 10^5 thymic cells/well $\pm$ SD. Numbers in parentheses are of cells recovered per thymus $\pm$ SD. P values were calculated by Student's t test for a given group compared to TF5 treatment.

obtained only in TF5-treated groups. Mice treated with kidney fraction 5 had some degree of response only on Day 14 after irradiation. This parallels the decline in cell numbers in the thymus. The increase in response was not consistent as it was not noted on Day 16. The irradiated group only responded to IL-1 on Day 16 after irradiation. In conclusion, (a) C57Bl/6 mice, as previously indicated (28), are low responders to IL-1, (b) irradiation of these mice results in a further decline of IL-1-responsive cells, which persist despite regeneration of the thymic cellularity nearly to control levels, and (c) administration of TF5 restores to normal the responsiveness to IL-1 on a per cell basis. This increase parallels the increase in cellularity. Treatment with kidney fraction 5 did not induce similar effects.

(3) DBA/1. Mice of this strain were evaluated since in previous experiments TF5 did not affect their resistance to infection with *C. albicans* and their *in vivo* release of IFN-γ and MIF. Animals of two different ages were used to determine whether the responses to TF5 are age dependent. The particular choice of ages, 10 weeks and 5 months, was based on the previous observation (27) that 8- to 10-week-old CDF_1 mice had maximal responses to IL-1 and 18-week-old mice had greatly reduced responses to purified IL-1. The lower responses in older animals may be an indication of reduced levels of immunocompetent T cells in the thymus, possibly as a result of reduced effectiveness of thymic hormones.

(a) Thymocytes from 10-week-old mice at 6, 7, and 9 days after irradiation when treated with TF5 showed consistently higher responses (Table 3). The thymocyte responses were lower in animals treated with kidney fraction 5 or irradiated only. Although lower number of cells per thymus were recovered in kidney fraction 5- and TF5-treated animals than in irradiated only mice at 6 and 7 days after irradiation, similar numbers of cells were recovered in TF5-treated group and irradiated group at 9 days after irradiation. Depletion of cells, therefore, in the thymuses of TF5-treated mice does not account for the apparent difference in the level of IL-1 reactive cells.

(b) The responses of thymocytes from 5-month-old mice evaluated in two series of experiments are summarized in Fig. 1. None of the irradiated experimental

TABLE 3

Effect of Thymosin Fraction 5 on Comitogenic Response of Thymocytes from 10-Weeks-Old DBA/1 Mice to IL-1

	D + 6		D + 7		D + 9	
Treatment	IL-1	Control	IL-1	Control	IL-1	Control
Thymosin fraction 5	5573 ± 757	1102 ± 333	3158 ± 775	1051 ± 241	3270 ± 1065	1349 ± 521
	(2.7×10^7)		(5.5×10^7)		(6.0×10^7)	
Kidney fraction 5	1197 ± 176	769 ± 93	1067 ± 91	522 ± 44	979 ± 131	671 ± 162
	(3.3×10^7)		(5.9×10^7)		(4.4×10^7)	
	$P < 0.002$		$P < 0.03$		$P < 0.02$	
Irradiated only	1678 ± 501	829 ± 89	1055 ± 51	744 ± 111	1324 ± 224	1110 ± 240
	(5.1×10^7)		(8.0×10^7)		(6.0×10^7)	
	$P < 0.01$		$P < 0.02$		$P < 0.03$	
Normal, non-irradiated	2457 ± 430	828 ± 286	1718 ± 206	714 ± 186	9276 ± 910	1529 ± 143
	(1.7×10^8)		(4.0×10^8)		(3.3×10^8)	

Note. See Table 1 for details. 3.0×10^6 cells/well in all groups. P as in Table 2.

groups showed the presence of IL-1-responsive cells at the cell concentrations used in the assay. The cellularity of the thymuses increased with no apparent influence of treatment. The only possible benefit of treatment may be observed on Day 20, when the numbers of cells in the thymuses of TF5-treated group were threefold that of the control normal animals. However, enhanced cellularity was also observed in animals which were irradiated only. Thus we can conclude that TF5, although effective in thymic recovery of younger animals, did not affect the recovery of the thymus in older animals. Cell proliferation, however, takes place in these thymuses following radiation injury, and at 12 days the numbers of thymocytes in irradiated mice nears that of normal mice.

C3H/HeJ. Mice of this strain, 5–6 months old, were used for comparison with TF5 unresponsive DBA/1 mice of the same age (Fig. 1). Two series of the separate experiments are summarized in Table 4. Since on Day 6 and 7 after irradiation the recovery of cells in individual groups (6 mice in each group) was low, the cell concentrations were reduced to 5×10^5 cells/well on Day 6 and to 7×10^5 cells/well on Day 7. It is evident from Table 4 that on Days 6, 7, and 9 after irradiation only thymic cells from TF5 treated mice responded to IL-1 with increased proliferation. In contrast, equal cell concentrations from irradiated only, irradiated and kidney fraction 5-treated, and non-irradiated normal mice were not responsive to IL-1. In conclusion, unlike DBA/1 mice, 5- to 6-month-old C3H/HeJ mice respond to TF5 treatment with enhanced responses of the cells in the thymus to IL-1.

DISCUSSION

The experimental results reported here address three questions: (1) are the cells repopulating the thymus in the early postirradiation phase immunologically com-

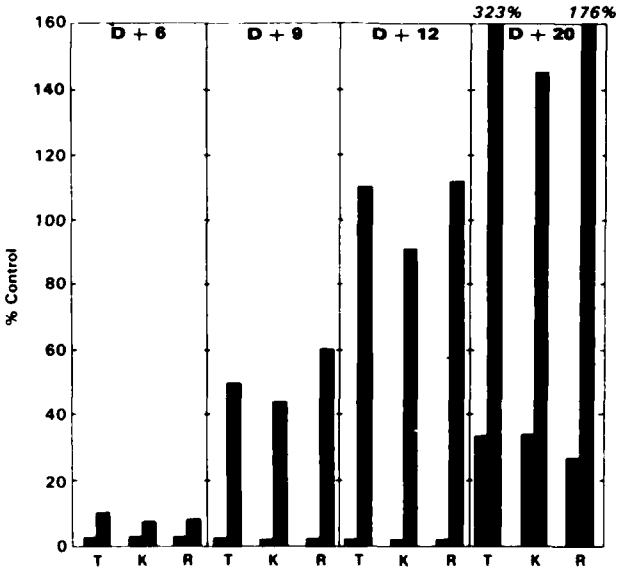

FIG. 1. Effect of thymosin fraction 5 on comitogenic response of thymocytes from 5-month-old DBA/1 mice to IL-1. Results expressed in percentage of control responses: (▩)-cpm (3×10^6 cells/well), (■) cells/thymus (normal cells/thymus, $6.8 \pm 2.8 \times 10^7$). T, thymosin fraction 5 treated; K, kidney fraction 5 treated; R, irradiated only.

TABLE 4

Effect of Thymosin Fraction 5 on Comitogenic Response of Thymocytes from 5-Months-Old C_3H/HeJ Mice to IL-1

	D + 6		D + 7		D + 9	
Treatment	IL-1	Control	IL-1	Control	IL-1	Control
Thymosin fraction 5	*4583 ± 2112 (1.0×10^6)	753 ± 79	**1938 ± 174 (3.1×10^6)	432 ± 188	†6324 ± 493 (6.5×10^7)	1776 ± 619
Kidney fraction 5	*1367 ± 1000 (1.0×10^6) $P < 0.02$	736 ± 356	**1225 ± 203 (4.1×10^6) $P < 0.005$	540 ± 328	†1410 ± 95 (5.4×10^7) $P < 0.01$	1607 ± 630
Irradiated only	*1959 ± 2050 (1.0×10^6) $P < 0.05$	1713 ± 1061	**937 ± 204 (4.1×10^6) $P < 0.05$	540 ± 255	†1349 ± 413 (5.1×10^7) $P < 0.001$	1683 ± 198
Normal, non-irradiated	†4096 ± 1391 (7.7×10^7)	1499 ± 801	†6162 ± 1189 (1.9×10^8)	937 ± 101	†3752 ± 414 (1.5×10^8)	1905 ± 249
	*685 ± 187	475 ± 112	**264 ± 120	226 ± 77		

Note. See Tables 1 and 2 for details. The number of cells/well were: *5×10^5, **7×10^5, †3×10^6. IL-1 concentration was 2 (D + 6) or 5 (D + 9) units/ml.

petent, (2) can thymic hormones exert an effect on these cells (or change their composition), and (3) can genetic factors influence the effect exerted by thymic hormones in irradiated mice.

Thymocytes from all of the strains examined, after irradiation with 450 cGy showed reduction in IL-1 responsiveness throughout the first phase of recovery. Earlier studies by Takada *et al.* (19), using mitotic index and thymus weight from mice irradiated with 400 cGy concluded that thymus regeneration is a biphasic process. Within 24 hr after irradiation, a precipitous drop in mitosis and in thymic weight developed which was followed by nearly full recovery beginning on Days 5–7 until Day 12. Following this day a second drop in thymic cellularity was observed which lasted until Day 20. Our observation on the recovery of cells in the thymus parallels these findings. The mechanism of this biphasic pattern of thymus repopulation remains speculative.

The immunologic competence of the cells initially repopulating the thymus is controversial. A number of studies concluded that radiation-resistant cells repopulating the thymus are immunocompetent. Blomgren and Anderson (29) compared cells from normal thymuses with corticosteroid-resistant cells (CRC) for their radiation resistance. They observed that corticosteroid treatment enriched the radiation-resistant cells from 4% in normal mice to 50% CRC populations, and concluded that these two populations may be similar. Studies by Konda *et al.* (30) examining the buoyant density and T-cell markers of CRC concluded that this population was similar to radioresistant cells present in the thymus 10 days after 880 cGy irradiation. Using 760 cGy irradiated A/J mice, Kadish and Basch demonstrated that cells recovered 9 days after irradiation had enhanced reactivity to Con A and PHA (31). The histology of cortical and medullary regions of the thymus 10 days after irradiation with 750 cGy resembled that of a normal thymus (32). Therefore it has been proposed that the radioresistant cells in the thymus that repopulate the thymus

after irradiation present a population resembling the mature CRC. More recent studies on the phenotypes of cells present in the thymus within 12 days following irradiation demonstrated a relative sparsity of PNA^-, Lyt-1^+-2^- cells (33), presently recognized as the immunocompetent subpopulation that is responsive to IL-1 (21–24). Similarly, CTL-precursor cells were found 14 days after lethal irradiation and bone marrow transfer but were not detected at 7 days after irradiation (34). In another laboratory the frequency of CTL-precursor cells was about 50-fold lower for up to 12 days after irradiation in the thymuses of bone marrow reconstituted radiation chimeras (35). The same investigators also analyzed by flow microfluometry the Thy-1 phenotype of host-derived cells. Despite increasing numbers of Thy-1-bright cells (considered immunologically immature), cells weakly stained with Thy-1 (considered immunocompetent) were not detected at 10 days after irradiation.

Our own observations using IL-1 responsiveness as a measure of thymocyte immunocompetence indicates a reduction in these cells from Day 6 to 12 postirradiation with 450 cGy. Given that the number of cells recovered from the thymus at 6 days after irradiation represents about 15% of the number of cells in normal thymus and nears normal at 12 days, the frequency of these cells in the thymus must be greatly reduced. Together with the finding on reduced frequency of CTL precursor cells (34, 35) and the scarcity of PNA^-, Lyt-1^+-2^-, weakly Thy-1 stained cells repopulating the thymus after irradiation (33, 35) the degree of maturation of radioresistant cells and the types of proliferating cells in a regenerating thymus need to be reevaluated.

There have been numerous demonstrations that various thymic hormones preparations are effective in treatment of immunodeficiencies (5–7, 36–39). The capacity of these hormones to promote maturation, proliferation, and marker acquisition of T cells in bone marrow or in spleen has been reported (8–10). However, the majority of the successful experiments demonstrating the effect of thymic hormones have been conducted *in vivo* despite the fact that most of the *in vitro* experiments use doses of the hormones manyfold higher than the doses used in the living animal (40). Possibly the action of this hormone is amplified *in vivo* via a mediating mechanism absent in the *in vitro* systems. The relatively narrow range of optimal doses necessary to achieve beneficial *in vivo* effects as well as necessity for daily injections of the hormone represent some of the not yet understood complexities of the system.

Administration of KF5 in doses equal to TF5 did not induce enhanced responsiveness to IL-1 in thymic cells from irradiated mice. Indications exist that certain thymic hormones may be present in organs other than the thymus. However, their levels in the kidney compared with the thymus are greatly reduced (41).

Although our results clearly demonstrate an enhancement of IL-1 responsiveness following administration of TF5 to irradiated mice, the mechanism of this enhancement remains unclear. Several possibilities should be considered. (a) Stimulation of the traffic of the bone-marrow-derived T-precursor cells into the thymus, (b) promotion of maturation of intrathymic cells, and (c) enrichment for IL-1-reactive cells as a result of selective depletion by thymic hormones of immunologically immature cells present in the thymus. The latter possibility does not seem likely as the recovery of cells per thymus on a given day does not vary much between the different experimental groups. Treatment with TF5 enhanced the level of IL-1 responsiveness in all four strains examined when the age of the mice was 10–12

weeks. The role of genetic factors is suggested by the finding that 5- to 6-month-old C3H/HeJ mice responded to treatment (Table 4) while DBA/1 mice of the same age did not respond (Fig. 1). This difference parallels the previously observed effect of TF5 in these two strains when resistance to *C. albicans* and *in vivo* release of IFN-γ and MIF were compared (17, 18). The same two mouse strains also varied in their responses to IL-1. Three-month-old C3H/HeJ mice had 10-fold higher response than 10-week-old DBA/1 mice (data not presented). Although at 5 months the response of normal C3H/HeJ mice to IL-1 had declined, it was still at least 2- to 3-fold higher than the response of thymocytes from 5-month-old DBA/1 mice. This apparent difference to a comitogenic effect of IL-1 may be a reflection of differences in the percentages of mature, immunocompetent cells in the thymuses of these strains. For example, the percentage of medullary PNA^- cells differed from 14.6% in CBA mice to 9.5% in C57Bl/6 mice (1). Perhaps these differences in the numbers of immunocompetent cells in the thymuses of different strains may be the result of differences in the levels of endogenous thymic hormones or of cell responses to thymic hormones. The responsiveness of 5- to 6-month-old C3H/HeJ mice to thymic hormones may be the reason for this strain's high level of IL-1 responses, and therefore the greater number of immunocompetent cells in the thymus. The unresponsiveness of the DBA/1 mice of the same age would result in lower numbers of IL-1 responsive cells in the thymus of this strain as observed in the present study. As reagents to evaluate thymic hormones levels in mice of different strains become available, this hypotheses may be examined.

ACKNOWLEDGMENTS

We thank Dr. Joost J. Oppenheim for challenging discussions and revision of the manuscript, Mr. W. E. Jackson for statistical analysis of the data and Mrs. Betty S. Markovich for typing the manuscript.

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Int. J. Immunopharmac., Vol. 7, No. 6, pp. 923–932, 1985.
Printed in Great Britain.

International Society for Immunopharmacology.

STIMULATED HEMOPOIESIS AND ENHANCED SURVIVAL FOLLOWING GLUCAN TREATMENT IN SUBLETHALLY AND LETHALLY IRRADIATED MICE

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(*Received* 12 *February* 1985 *and in final form* 16 *May* 1985)

Abstract — Hemopoietic effects of the reticuloendothelial agent glucan were assayed in normal mice and in mice hemopoietically depleted by exposure to ^{60}Co radiation. In normal mice, glucan administration increased the content of bone marrow and splenic transplantable pluripotent hemopoietic stem cells (CFU-s), committed granulocyte-macrophage progenitor cells (GM-CFC), and pure macrophage progenitor cells (M-CFC). Erythroid progenitor cells (CFU-e) were increased only in the spleen. In sublethally irradiated mice (650 rads), glucan increased the number of endogenous pluripotent hemopoietic stem cells (E-CFU) when administered either before or after irradiation. The most pronounced effects were observed when glucan was administered 1 day before, 1 h before, or 1 h after irradiation. In addition, the administration of glucan before lethal irradiation (900 rads) enhanced survival. The most significant results were seen when glucan was administered 1 day prior to irradiation. The possibility of using agents such as glucan to enhance hemopoietic reconstitution and prevent septicemia following chemotherapy and/or radiotherapy is discussed.

Glucan, a B-1,3 polyglucose, has been shown to modulate reticuloendothelial and immune responses. For instance, it has been shown that macrophage proliferation, phagocytosis, adherence, and lysozyme synthesis as well as primary and secondary antibody responses, cell-mediated immune responses, anti-tumor responses, and anti-bacterial, -fungal, -viral, and -parasitic responses are enhanced following glucan administration (Riggi & DiLuzio, 1961; Wooles & DiLuzio, 1963; DiLuzio, 1967; DiLuzio, Pisano & Saba, 1970; Kokosis, Williams, Cook & DiLuzio, 1978; DiLuzio, Williams, McNamee, Edwards & Kilahama, 1979; Reynolds, Kastello, Harrington, Crabbs, Peters, Jemski, Scott & DiLuzio, 1980; Cook, Holbrook & Parker, 1980; DiLuzio, 1983). In addition, glucan has been shown to alter hemopoietic proliferation and differentiation. Specifically, increased numbers of not only granulocyte-macrophage (GM-CFC), pure macrophage (M-CFC), and erythroid (CFU-e and BFU-e) progenitor cells but also pluripotent hemopoietic stem cells that give rise to these progenitors (CFU-S) have been observed following glucan administration (Burgaleta & Golde, 1977; Patchen & Lotzova, 1980; Patchen & MacVittie, 1983a). The multiple effects of glucan on the reticuloendothelial, immune, and hemopoietic systems thus make glucan an intriguing candidate for therapeutic use in instances of radiation-induced and/or chemical-induced hemopoietic and immune depression.

The purposes of this study were to (a) delineate the temporal hemopoietic responses elicited by a glucan dose known to stimulate reticuloendothelial and immune responses in normal mice, (b) determine the feasibility of using glucan to enhance recovery from radiation-induced hemopoietic depletion, and (c) determine the feasibility of using glucan to enhance survival in irradiated mice.

EXPERIMENTAL PROCEDURES

Mice

In all experiments, 10–12 week old female C_3H/HeN mice (Charles River Laboratories, Willmington, MA) were used. Animals were maintained on a 6 a.m. to 6 p.m. light–dark cycle. Wayne Lab Blox and hyperchlorinated water were

available *ad libitum*. All mice were quarantined and acclimated to laboratory conditions for at least 2 weeks before experimentation. During this time, the mice were examined and found to be free of lesions of murine pneumonia complex and oropharyngeal *Pseudomonas* sp.

Glucan

Particulate, endotoxin-free glucan (glucan-P) was obtained from Dr. N. R. DiLuzio (Tulane University School of Medicine, New Orleans, LA) and was prepared according to DiLuzio's modification (DiLuzio *et al.*, 1979) of Hassid's original procedure (Hassid, Joslyn & McCready, 1941). Sterile saline was used to dilute glucan-P to either 3.0 mg or 0.8 mg per ml. Mice were then i.v. injected via the lateral tail veins with a 0.5 ml volume of the glucan preparations yielding either 1.5 mg or 0.4 mg per mouse. Normal control mice (not irradiated and not glucan-treated) and radiation control mice (irradiated but not glucan-treated) were injected with an equivalent volume of sterile saline.

Cell suspensions

Each cell suspension represented the pool of tissues from three mice. Cells were flushed from femurs with 3 ml of Hank's Balanced Salt Solution (HBSS) containing 5% heat-inactivated fetal bovine serum (HIFBS). Spleens were pressed through a stainless-steel mesh screen, and the cells were washed from the screen with 6 ml of HBSS plus 5% HIFBS. The total number of nucleated cells in each suspension was determined by counting the cells on a hemocytometer.

Irradiation

Bilateral total-body irradiation administered from the AFRRI ^{60}Co source at a dose rate of 40 rads/min was used in all radiation experiments.

Spleen colony-forming unit (CFU) assays

Spleen colony-forming units (CFU) have been shown to arise from the clonal proliferation of pluripotent hemopoietic stem cells. Exogenous spleen colony-forming units (CFU-s) were evaluated by the method of Till & McCulloch (1961). Recipient mice were exposed to 900 rads of total-body irradiation in order to completely eradicate endogenous hemopoietic stem cells. Three to 5 h later, 5×10^4 bone marrow or 5×10^5 spleen cells were i.v. injected into the irradiated recipients. Ten days after transplantation, the recipients were euthanized and their spleens removed. The spleens were fixed in Bouin's solution, and the number of grossly visible spleen colonies were counted. Endogenous spleen colony-forming units (E-CFU) were also evaluated by a method of Till & McCulloch (1963). Mice were exposed to a 650 rads dose of total-body irradiation in order to only partially oblate endogenous hemopoietic stem cells. Ten days after irradiation, the spleens were removed and fixed in Bouin's solution. Then the spleen colonies formed by the proliferation of surviving endogenous hemopoietic cells were counted.

Granulocyte-macrophage colony-forming cell (GM-CFC) and macrophage colony-forming cell (M-CFC) assays

Hemopoietic progenitor cells committed to the granulocyte-macrophage lineage (GM-CFC) were assayed by MacVittie's modification (1979) of the semi-solid agar technique originally described by Bradley & Metcalf (1966) and Pluznik & Sachs (1965). The upper agar-medium mixture for cell suspensions consisted of equal volumes of 0.66% agar and double-strength supplemented CMRL 1066 medium. The CMRL 1066 was supplemented with final concentrations of 10% HIFBS, 5% trypticase soy broth, 5% heat-inactivated horse serum, antibiotics and L-asparagine (30 μg/ml). The agar-medium mixture for the lower feeder layer consisted of equal volumes of 1.0% agar and supplemented double-strength CMRL 1066. Both pregnant mouse uterine extract (PMUE) (2.5% v/v) and mouse L-cell-conditioned medium (LCM) (13% v/v) were added to each 1 ml feeder layer as sources of colony-stimulating activity (CSA). Colonies (>50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 7.5% CO_2. Hemopoietic progenitor cells committed only to the macrophage lineage (M-CFC) were assayed by the technique described by MacVittie & Provaznik (1978). Double-layer agar cultures were prepared as in the GM-CFC assay except that no CSA was initially incorporated into feeder layers. This initial absence of CSA eliminated the proliferation of GM-CFC. Two days after the cells were originally plated, CSA was added by incorporating PMUE and LCM into a mixture of supplemented CMRL 1066 containing a final concentration of 0.33% agar, and pipetting 0.5 ml of this mixture on top of the previously cultured cells. Cultures were incubated for an additional 25 days at 37°C in a humidified atmosphere containing 7.5% CO_2, before scoring colony formation.

Erythroid colony-forming-unit (CFU-e) assay

Bone marrow and splenic hemopoietic progenitor cells committed to the erythroid lineage (CFU-e) were assayed by a modification of the original CFU-e plasma clot technique described by Stephenson, Axelrad, McLeod & Shreeve (1971). Each ingredient was either reconstituted or diluted with supplemented alpha medium (SAM) (Weinberg, McCarthy, MacVittie & Baum, 1981). Briefly, to make 1 ml of the plasma clot suspension, the following were mixed: 0.1 ml cells (5×10^5 nucleated cells), 0.3 ml HIFBS, 0.1 ml 25% beef embryo extract, 0.1 ml 10% bovine serum albumin, 0.1 ml (0.02 mg) L-asparagine, 0.1 ml 10^{-3}M 2-mercaptoethanol, 0.1 ml erythropoietin (Ep), and 0.1 ml of 37°C bovine citrated plasma. Immediately, 0.1 ml of the mixture was pipetted into each of six microtiter wells. Step III anemic sheep plasma (Connaught Labs, Inc., Swiftwater, PA) was used as the source of Ep. Bone marrow and splenic CFU-e clot suspensions contained 0.25 and 0.5 untis Ep/ml, respectively. Control clots contained SAM in place of Ep. After incubation at 37°C in a humidified atmosphere containing 5% CO_2 in air for 2 days, plasma clots were harvested, fixed with 5% gluteraldehyde, and stained with benzidine and giemsa (McLeod, Shreeve & Axelrad, 1979). A CFU-e was defined as an individual aggregate of eight or more benzidine-positive cells.

Survival studies

Mice used in survival studies were exposed to 900 rads of total-body irradiation, and their survival was checked daily for a period of 30 days.

RESULTS

Temporal hemopoietic effects of glucan-P in normal mice

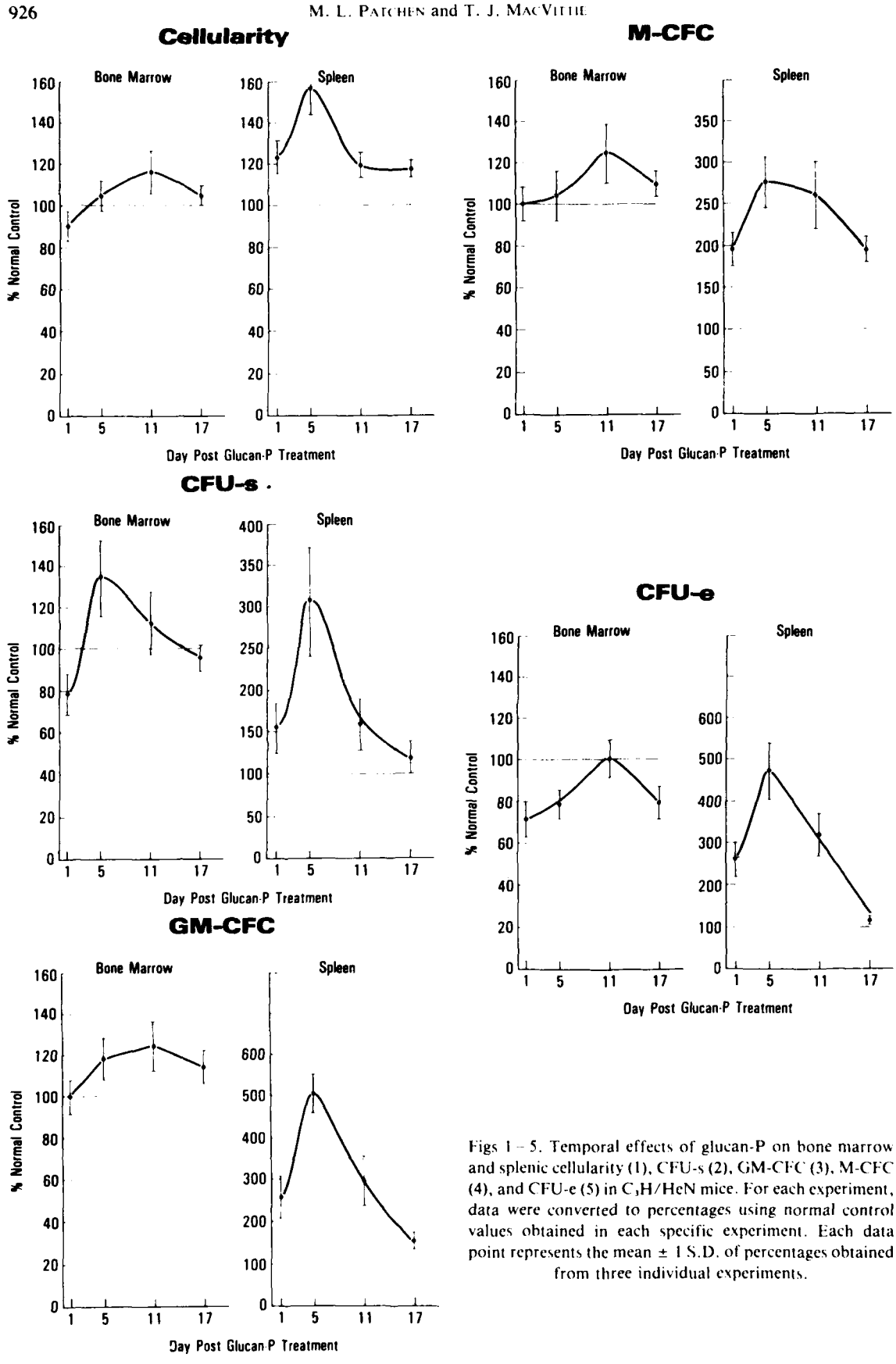
Figures 1–5 illustrate the temporal effects of 1.5 mg of glucan-P on bone marrow and splenic cellularity, CFU-s, GM-CFC, M-CFC, and CFU-e responses in normal mice. Except for bone marrow CFU-e, the absolute numbers of these various hemopoietic elements all increased following glucan-P administration. Peak splenic responses were consistently observed 5 days after glucan-P treatment. The bone marrow CFU-s response also peaked on day 5 post-treatment; however, peak marrow GM-CFC and M-CFC responses did not occur until day 11 post-treatment. The bone marrow hemopoietic responses were also much less dramatic than the splenic hemopoietic responses, e.g. 115–135% of normal control values in the bone marrow vs 270–500% of normal control values in the spleen. Interestingly, bone marrow CFU-e values never rose above normal control values and, in fact, were in most instances lower than control values. This erythropoietic depression, however, was only temporary, and by 30 days post-treatment, hematocrit values in glucan-P-treated mice had returned to normal control values (Table 1).

Glucan-P's effect on enhancing recovery from radiation-induced hemopoietic depletion

The endogeneous spleen colony assay was used to screen glucan-P for its ability to enhance hemopoietic pluripotent stem cell recovery when administered before or after a hemopoietically compromising dose of irradiation. Table 2 presents data from experiments in which glucan-P was administered at 17, 11, 5 and 1 days or 1 h before or after a 650 rads dose of ^{60}Co irradiation. Because an enhanced E-CFU response was anticipated, both a 1.5 mg and a 0.4 mg glucan-P dose were used in order to insure that discrete countable E-CFU colonies could be obtained. Glucan-P was quite effective at enhancing E-CFU numbers even when administered as long as 17 days before irradiation. However, as the interval between glucan-P treatment and irradiation was shortened, the E-CFU response became more pronounced, with almost equally dramatic effects being observed with glucan-P administered at either 1 day or 1 h before irradiation. Although not as pronounced as preirradiation glucan-P treatment, postirradiation glucan-P treatment was also quite effective at enhancing E-CFU colony formation. In fact, glucan-P given 1 h after irradiation was almost as effective at enhancing E-CFU colony formation as was glucan-P administered 1 h before irradiation.

Glucan-P's effect on survival of irradiated mice

Because of glucan-P's ability to enhance hemopoietic recovery when administered both before and after irradiation, the ability of this agent to also enhance survival in otherwise lethally irradiated animals was next investigated. Based on the E-CFU results, glucan-P was administered either 1 day or 1 h before or 1 h after irradiation. As illustrated in Fig. 6, 1.5 mg of glucan-P administered 1 day before an otherwise lethal dose of irradiation significantly enhanced survival. Thirty days postirradiation, 51% of the 1.5-mg glucan-P-treated mice were alive. Although the protocol for these studies included only



Figs 1 – 5. Temporal effects of glucan-P on bone marrow and splenic cellularity (1), CFU-s (2), GM-CFC (3), M-CFC (4), and CFU-e (5) in C_3H/HeN mice. For each experiment, data were converted to percentages using normal control values obtained in each specific experiment. Each data point represents the mean ± 1 S.D. of percentages obtained from three individual experiments.

Table 1. Hematocrit values in glucan-P treated mice

	Day post glucan-P treatment							
	1	5	11	17	30	60	90	120
Hematocrit value % of normal control	102±3	98±2	83±3	89±4	99±3	101±3	100±2	102±3

Table 2. Effect of pre- and post-irradiation glucan-P treatment on E-CFU

	Pre 650 rads					Post 650 rads				
Time of glucan-P injection (days)	17	11	5	1	1 h	1 h	1	5	11	17
E-CFU (% radiation control value*)										
0.4 mg (n = 24)	174±29	261±45	306±52	590±90	548±66	539±60	408±48	141±48	†	†
1.5 mg (n = 24)	326±26	442±45	990±110	1503±177	1448±174	1355±139	447±95	260±35	†	†

*Radiation control E-CFU values for −17, −11, −5, −1, −1h, +1h, +1, and +5 days were 1.9±0.2, 2.0±0.3, 2.1±0.4, 1.9±0.4, 2.2±0.6, 1.7±0.7, 2.3±0.7 and 10.0±1.8, respectively.
†Not quantifiable due to confluent colony growth.

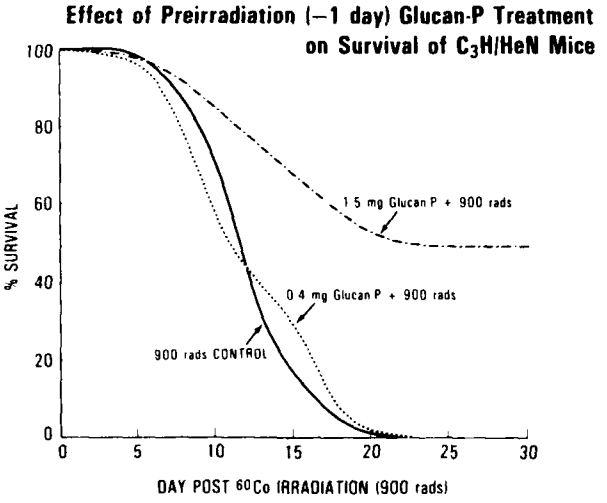


Fig. 6. Effects of two doses of Glucan-P on 30 day survival of C_3H/HeN mice when injected 1 day before 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 5 – 15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 83 mice, and 0.4 mg glucan-P data are based on 56 mice.

a 30 day survival, 49% of these irradiated mice treated with 1.5 mg of glucan-P actually exhibited long-term survival and were ultimately euthanized 12 - 13 months after irradiation. By contrast, the lower 0.4-mg glucan-P dose was not effective at enhancing survival in similarly irradiated mice. Similar to radiation controls, none of these mice survived beyond 21 days postirradiation. Figure 7 illustrates the survival effects of glucan-P administered 1 h before lethal irradiation. As can be

seen, survival in mice receiving 0.4 mg of glucan-P did not differ from radiation controls. However, survival in mice receiving the 1.5 mg glucan-P dose was slightly prolonged, even though long-term survival (i.e. ≥ 30 days) was not different from that of radiation controls. Although 1.5 mg glucan-P treatment did exhibit some positive effects on survival when administered prior to irradiation, it was not effective when administered after irradiation (Figure 8). In fact, when administered postirradiation, glucan-P actually induced death more rapidly than in radiation controls. This effect was dose-dependent, with the higher 1.5 mg glucan-P dose being more detrimental than the lower 0.4 mg glucan-P dose.

DISCUSSION

Agents capable of regulating (and in particular stimulating) hemopoietic proliferation and differentiation at the stem cell level have a variety of potential benefits. One such benefit would be to reduce lethality attributed to the hemopoietic syndrome following irradiation and/or chemotherapy. Because reticuloendothelial and immune reconstitution are critically important in combating the secondary septicemia associated with hemopoietic depletion, (Hammond, Tompkins & Miller, 1954; Benacerraf, 1960; Cronkite & Bond, 1960; Collins, 1979; Broerse & MacVittie, 1984) we specifically evaluated the hemopoietic effects of glucan-P in the dose range that has been shown to elicit enhanced reticuloendothelial and immune responses.

These studies have clearly demonstrated several effects of glucan-P on normal murine hemopoiesis and on hemopoiesis and survival in irradiated mice. First, our results concerning the temporal effects of 1.5 mg of glucan-P on splenic and bone marrow CFU-s, GM-CFC, M-CFC and CFU-e clearly confirm previous studies suggesting that particulate glucan, in a dose-dependent manner, can enhance hemopoiesis in normal mice (Burgaleta & Golde, 1977; Patchen & Lotzova, 1980; Patchen & MacVittie, 1983a,b). In the studies presented here, stem and progenitor cell hemopoietic responses were evident as early as 1 day post glucan-P treatment, usually peaked on days 5 – 11 post-treatment, and had either returned to or were declining toward normal control values by 17 days post-treatment. Bone marrow erythropoiesis was the only hemopoietic response not enhanced. However, the tremendous increase in splenic erythropoiesis apparently compensated for the decreased bone marrow erythropoiesis, since only a transient anemia was apparent in glucan-P-treated mice.

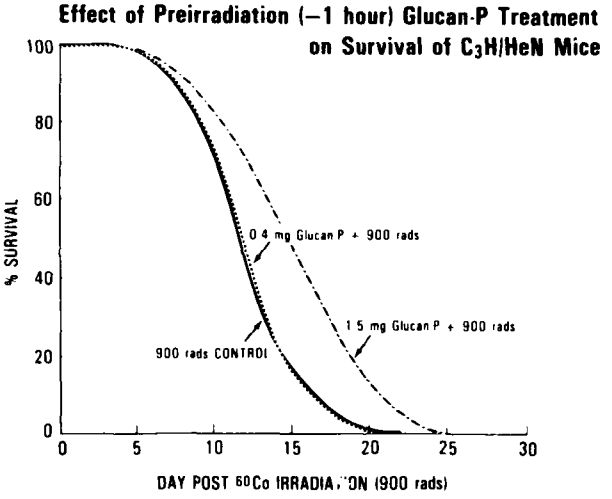


Fig. 7. Effects of two doses of glucan-P on 30 day survival of C3H/HeN mice when injected 1 h before 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 4 – 15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 59 mice, and 0.4 mg glucan-P data are based on 54 mice.

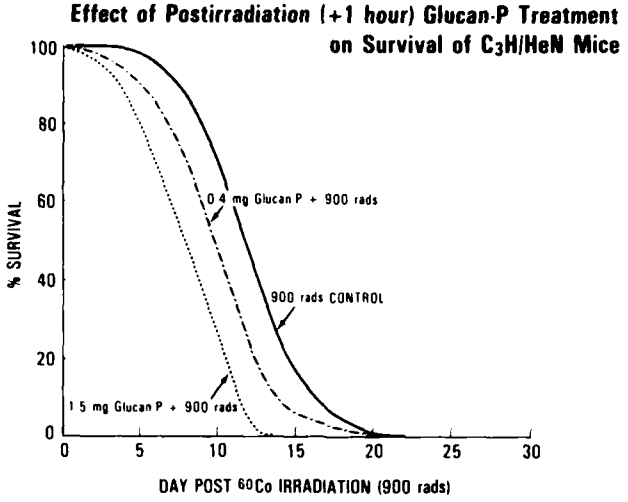


Fig. 8. Effects of two doses of glucan-P on 30 day survival of C_3H/HeN mice when injected 1 h after 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 3 – 15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 67 mice, and 0.4 mg glucan-P data are based on 30 mice.

Second, the ability of glucan-P to enhance hemopoietic recovery in irradiated mice was also demonstrated. In particular, it was shown that glucan-P could significantly increase pluripotent hemopoietic stem cell numbers (as measured by the E-CFU assay) in partially hemopoietically depleted mice if injected either before or after irradiation. The most dramatic increases were observed with glucan-P administered either 1 day before, 1 h before, or 1 h after irradiation. In any given experiment, glucan-P treatment 1 day before irradiation was slightly more enhancing than treatment 1 h before irradiation, and treatment 1 h before irradiation was slightly more enhancing than treatment 1 h after irradiation.

Finally, the fact that glucan-P administration could significantly influence survival following higher (lethal) doses of irradiation was also demonstrated. Specifically, 1.5 mg of glucan-P, administered 1 day before irradiation, significantly increased long-term survival (i.e. $\geq$ 30 days), and the same dose of glucan-P administered 1 h before irradiation marginally increased short-term survival (i.e. $<$ 30 days). However, when glucan-P was injected 1 h post-irradiation, not only was survival not increased, but it actually decreased compared to radiation controls.

Over the years a variety of other substances have also been shown to alter survival in irradiated mice (Smith, Smith, Andrews & Grenan, 1955; Smith, Alderman & Gillespie, 1957; Boggs, Marsh, Chervenick, Cartwright & Wintrobe, 1968; Mori & Nakamura, 1970). The substances used have been as diverse as ground glass, carbon particles, non-species related serum, and endotoxin. Almost all these substances produced the best "radioprotective" effect in mice when administered 1 day before irradiation. However, in addition to the timing of the injection, the route of injection was also shown to influence survival results. The survival results we have obtained with i.v. -1 day glucan-P administration in general, correlate with previous studies utilizing various other intravenously administered substances. Even with respect to post-irradiation glucan-P treatment decreasing survival (rather than increasing or not changing survival), a similar phenomenon has been observed with post-irradiation i.v. injection of endotoxin. For example, if either glucan-P or endotoxin are injected into mice 2 – 3 days after 900 rads of irradiation, all animals die almost immediately (Smith *et al.* 1957; Patchen, unpublished observations). Since our glucan-P preparation was endotoxin-free (as determined by the Limulus Lysate procedure), our decreased survival could not have been due to endotoxin contamination in our glucan-P. Rather, we suspect that the decreased survival seen with post-irradiation administration of glucan-P and endotoxin is a more general phenomenon probably inducible by other

agents as well. Interestingly, we have also been working with a different glucan preparation (glucan-F, a soluble B-1, 3 glucan), which is capable of producing the same dramatic survival-enhancing effects as glucan-P or endotoxin when administered 1 day before irradiation, but does not decrease survival (and, in fact, is even slightly radioprotective) when administered after irradiation (Patchen *et al.*, unpublished results). Studies to further evaluate the mechanisms of the differential effects of pre- and post-irradiation glucan-P treatments on survival are currently being conducted in our laboratory.

A variety of mechanisms have been hypothesized to explain the capabilities of numerous "radioprotective" agents (e.g. ground glass, carbon particles, endotoxin, etc.). Most of these agents are "radioprotective" only in the hemopoietic syndrome radiation dose range. Thus, it is not surprising that hemopoietic protection and/or enhanced hemopoietic reconstitution followed by enhanced resistance to endogenous pathogens have been shown to occur following administration of these agents. Since the E-CFU assay measures post-irradiation recovery of endogenous pluripotent hemopoietic stem cells (i.e. the cells from which all other mature cells comprising the blood, immune, and reticuloendothelial systems ultimately arise), a direct correlation has been suggested between an agent's hemopoietic-enhancing potential (as measured by the E-CFU assay) and its survival-enhancing potential in the hemopoietic syndrome radiation dose range (Smith, Budd & Cornfield, 1966; Kinnamon, Ketterling, Stampfli & Grenan, 1980). Based on the differences we observed in E-CFU numbers and survival data produced by −1 day, −1 h, and +1 h glucan-P treatment, our data also seem to suggest such a correlation. However, in spite of the fact that −1 day glucan-P-treated mice did exhibit slightly higher endogenous hemopoietic stem cell numbers than −1 h glucan-P treated mice, and −1 h glucan-P treated mice did exhibit slightly higher endogenous hemopoietic stem cell numbers than +1 h glucan-P treated mice, all three treatment groups exhibited extremely elevated E-CFU numbers. Thus, it seems unlikely that the survival effects induced by these three glucan-P treatments would be so different if increased pluripotent hemopoietic stem cell numbers *alone* were responsible for enhanced survival. Instead, it may be possible that in mice treated with glucan-P 1 day before irradiation, pluripotent hemopoietic stem cells have enough of an opportunity to not only proliferate, but also to differentiate into progenitors capable of giving rise to the mature functional elements of the reticuloendothelial and immune systems necessary to contend with the surgence of endogenous pathogens that secondarily induce death 10–20 days post-irradiation in the 900 rads dose range (Hammond *et al.*, 1954; Benacerraf, 1960; Cronkite & Bond, 1960; Collins, 1979; Broerse & MacVittie, 1984). In fact, it has been shown that recovery of the differentiated progenitor cells that do give rise to granulocytes and macrophages is more accelerated in mice treated with glucan-P 1 day before irradiation than in mice treated with glucan-P just 1 h before or 1 h after irradiation (Patchen, 1983; Patchen, MacVittie & Wathen, in press).

In spite of the uncertainties concerning the mechanisms by which glucan-P mediates its effects, it is apparent that glucan-P is a potent hemopoietic stimulant in normal mice and in radiation-depleted mice and that glucan-P administered 1 day prior to otherwise lethal radiation in the hemopoietic syndrome dose range can significantly enhance survival. In addition, when compared to other historical "radioprotectors", glucan-P has the advantages of being nontoxic, nonpyrogenic, and ultimately it can be metabolized to glucose and utilized nutritionally as a food source (DiLuzio, 1983).

Glucan-P's hemopoietic and survival-enhancing capabilities, coupled with its ability to nonspecifically stimulate resistance to a variety of bacterial, viral, and fungal infections make glucan-P a primary candidate for use in instances of radiation-induced and/or chemical-induced life-threatening hemopoietic depletion.

Acknowledgements — The authors acknowledge the excellent technical assistance of Brenda Watkins and James Atkinson and the excellent editorial and typing skills of Junith Van Deusen and Gloria Contreras.

Supported by Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00132. The views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

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BEHAVIORAL AND NEURAL BIOLOGY 44, 364–373 (1985)

Intragastric Copper Sulfate Produces a More Reliable Conditioned Taste Aversion in Vagotomized Rats than in Intact Rats

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Although copper sulfate is an emetic stimulus, preliminary experiments failed to obtain a taste aversion in intact rats following intragastric administration as had been previously reported in the literature. Several experiments were therefore run to further investigate the capacity of intragastric copper sulfate to function as an unconditioned stimulus for taste aversion learning and the role of the vagus in mediating that learning. The results of the first series of experiments showed that intragastric administration of copper sulfate (5 mg/kg × $5H_2O$) was more effective in reliably producing a taste aversion in vagotomized rats than in sham-operated control rats. The second experiment examined the effects of area postrema lesions on the acquisition of a taste aversion produced by intragastrically administered copper sulfate in vagotomized rats. The results indicated that the taste aversion observed following treatment with intragastric copper sulfate in vagotomized rats could be prevented by lesions of the area postrema. The present results indicate that intragastric administration of copper sulfate is a more reliable unconditioned stimulus for taste aversion learning in vagotomized rats than in intact rats. It is not certain what factors might account for the discrepant results between the present experiments and previously published research.

When a novel tasting solution, such as saccharin or sucrose, is paired with a toxic unconditioned stimulus, such as lithium chloride (LiCl), the organism will avoid ingestion of that solution at a subsequent presentation. This avoidance behavior, called a conditioned taste aversion (CTA), is

[1] We acknowledge the support of the Computer Science Center facilities of the University of Maryland. A preliminary report of some of the data was presented at the 91st Meeting of the American Psychological Association, Anaheim, CA 1983. This research was conducted according to the principles described in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Research, National Research Council. Send requests for reprints to Dr. Rabin at Department of Psychology, University of Maryland Baltimore County, Catonsville, MD 21228.

0163-1047/85 $3.00

typically acquired in a single trial. In addition to LiCl, a CTA can also be produced by exposure to ionizing radiation (Smith, 1971) and by systemic treatment with a variety of toxic and nontoxic drugs (Garcia, Hankins, & Rusiniak, 1974; Goudie, 1979).

Because subdiaphragmatic vagotomy disrupts the acquisition of a CTA produced by intragastric or intraperitoneal administration of copper sulfate ($CuSO_4$), Coil, Rogers, Garcia, and Novin (1978) have proposed that a vagally mediated gastric irritation is the proximal unconditioned stimulus leading to CTA learning following systemic treatment with $CuSO_4$. Since exposing an organism to ionizing radiation also has a variety of effects on the gastrointestinal system, Hulse and Mizon (1967) have proposed that these radiation-induced changes in gastrointestinal system functioning contribute to the acquisition of a CTA following irradiation.

As part of an experiment on the effects of subdiaphragmatic vagotomy on the acquisition of a radiation-induced CTA (Rabin, Hunt, & Lee, 1983b), a group of intact rats was included which was treated with intragastric $CuSO_4$ as a procedural control. However, in contrast to the results obtained by Coil et al. (1978) these rats did not develop a CTA following intubation with $CuSO_4$. Several additional experiments were therefore run to attempt to determine the factors that might have affected the failure to obtain a CTA and the role of a vagally mediated gastric irritation in the acquisition of a CTA produced by intubation of $CuSO_4$.

GENERAL METHODS

The subjects for the various experiments were male Sprague–Dawley derived rats weighing 250–350 g at the start of the experiment. The animals were maintained in individual cages in a room with a 12:12 light:dark cycle. Food and water were continually available except as required by the experimental protocol.

Taste aversions were produced using a single-bottle procedure similar to that used by Coil et al. (1978). The subjects were first placed on a 23.5-h water deprivation schedule for 7–10 days during which water was available for 30 min each day during the early light phase of the diurnal cycle. On the three conditioning days the water bottle was replaced by a single bottle containing 10% sucrose for the 30-min drinking period. Following this, the rats were treated with intragastric $CuSO_4$ (5 mg/kg $\times$ $5H_2O$) or isotonic saline administered through an infant feeding tube. On the test days, the rats were again presented with a single bottle containing 10% sucrose, but were not treated with the unconditioned stimulus. In the 3- to 4-day interval between each conditioning and test day, the rats were maintained on the standard water-deprivation schedule with access to water for 30 min each day.

The data for all experimental days are presented as the percentage of the sucrose intake on the initial conditioning day. Primary data analysis was done with repeated measures analyses of variance. Where necessary, detailed comparisons between treatment groups were done using orthogonal comparisons (Keppel, 1973).

All surgical procedures were performed under anesthesia provided by combined injections of ketamine (120 mg/kg, ip) and pentobarbital (21 mg/kg, ip). Vagotomies were made using the procedures described by Martin, Rogers, Novin, and VanderWeele (1977). For the sham-vagotomized rats, the vagus was exposed, but not sectioned. After surgery, the rats were allowed to recover for 3–5 weeks before beginning the deprivation training. Lesions of the area postrema (AP) were made using thermal cauterization under direct visual control (Rabin, Hunt, & Lee, 1983a). In the sham-operated rats, the AP was exposed, but not cauterized. These animals were allowed a 2- to 3-week recovery period before beginning the next phase of the experiment.

At the conclusion of the experiments, the animals were sacrificed with pentobarbital (50 mg/kg, ip) to permit verification of the vagotomy and AP lesion. The vagotomies were verified either by determining the stomach weight/body weight ratios of the vagotomized rats, who show a significantly greater ratio than do control rats (Martin et al., 1977) or by recording the response of the stomach to electrical stimulation of the cervical vagus (Coil et al., 1978). For verification of the AP lesions, the brains of the rats were fixed in 10% formalin saline. Sections were then cut at 50 μm through the brainstem at the level of the AP and stained with thionin.

EXPERIMENT 1

In a series of experiments using intact rats in a two-bottle test, Rabin et al. (1983b) were not able to reliably obtain a CTA despite varying numbers of conditioning trials or varying doses. These results are not consistent with the results of Coil et al. (1978) who reported that intragastric $CuSO_4$ at a dose of 5 mg/kg produces a CTA which can be disrupted by sectioning the vagus. It may be that the single-bottle test or surgical procedures utilized in the successful experiments by Coil et al. (1978) contributed to the acquisition of a CTA following treatment with intragastric $CuSO_4$. The present experiment, therefore, was designed to partially replicate the procedures used by Coil et al. (1978) to determine whether under these conditions intragastric $CuSO_4$ would reliably lead to the acquisition of a CTA which could be disrupted by subdiaphragmatic vagotomy.

Methods. This experiment was run as two independent replications with 22 naive rats in the first and 30 naive rats in the second replication. In each replication, half the animals were vagotomized and half were sham-operated controls. In both replications, all subjects were administered

intragastric $CuSO_4$ (5 mg/kg × $5H_2O$) through an infant-feeding tube immediately following ingestion of a 10% sucrose solution on Days 1, 4, and 7 using a single-bottle design. Water was available for 30 min on the intervening days. In the first replication there was only a single test day on Day 10, while in the second, there were two test days on Days 10 and 14. The replications also differed in that different procedures were used to assess the completeness of the vagotomies: a comparison of stomach weight/body weight ratios in the first, and recording stomach contractions in response to stimulation of the cervical vagus in the second replication.

Results and Discussion. The results of the first replication are presented in Fig. 1 which indicates that the vagotomized animals showed a greater reduction in sucrose intake than did the control animals following treatment with intragastric $CuSO_4$. A two-way analysis of variance showed that the main effect for surgery for the comparison between the vagotomized and sham-operated rats was significant, $F(1, 20) = 8.21$, $p < .01$. Neither the main effect for the comparison across Days 4–10, $F(2, 40) = 0.20$, $p > .05$, nor the Surgery × Day interaction, $F(2, 40) = 1.64$, $p > .05$, was significant, indicating that the intragastric $CuSO_4$ produced a consistent decrease in sucrose intake in the vagotomized rats that was not observed in the controls.

Because these results are in direct contrast to those obtained by Coil et al. (1978), a second replication was run. These results are presented in Fig. 2. Unlike the results of the first replication, the analysis of variance indicated that the main effect for the comparison between the vagotomized and sham-operated animals did not reach the level of significance, $F(1,$

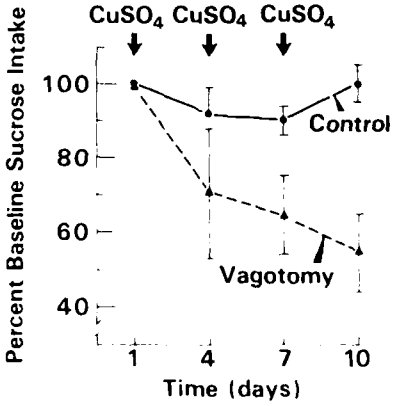

FIG. 1. First replication of the effects of repeated intragastric intubation of $CuSO_4$ (5 mg/kg × $5H_2O$) on sucrose intake using a single-bottle design in vagotomized and sham-operated control rats. The $CuSO_4$ was administered immediately after ingestion of the sucrose solution on Days 1, 4, and 7. Variance bars indicate the standard error of the mean.

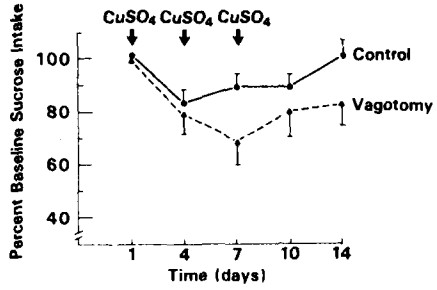

FIG. 2. Second replication of the effects of repeated intragastric intubation of $CuSO_4$ (5 mg/kg × $5H_2O$) on sucrose intake using a single-bottle design in vagotomized and control rats. Variance bars indicate the standard error of the mean.

28) = 3.43, $p < .10$, while the main effect for the comparison for day across Days 4–14 was significant, $F(3, 84) = 2.74$, $p < .05$. Since the Surgery × Day interaction was not significant, $F(3, 84) = 1.09$, $p > .10$, it would suggest that treatment with intragastric $CuSO_4$ produced a significant decrease in sucrose intake in both the vagotomized and control animals on at least several of the experimental days.

The results of these two replications partially fail to confirm the results of the experiments by Coil et al. (1978), who reported that vagotomy blocks the acquisition of a CTA produced by intragastrically administered $CuSO_4$. In neither of the replications did vagotomy reverse a $CuSO_4$-induced CTA observed only in the sham-operated control animals. Rather, the data would seem to indicate, at least based upon the results of the first experiment, that vagotomy may be a necessary condition for the reliable development of a CTA following treatment with intragastric $CuSO_4$. Although the results of the second replication seem to parallel those of the first replication, it is not clear why there were differences in results between the two replications. Examining Figs. 1 and 2 it seems that the $CuSO_4$ had a greater effect on the sucrose intake of the sham-operated controls in the second replication than in the first, although it is not clear why this occurred. Similarly, it seems that the vagotomy had a greater effect in the first replication than in the second. One possible explanation for this difference may be in the different methods used to validate the completeness of the vagotomy. Although there is no single test that is the best under all conditions (Louis-Sylvestre, 1983), it may be that the comparison of the stomach weight/body weight ratios used in the first replication provided a more reliable indicator of the completeness of the vagotomy than did the electrophysiological measure used in the second replication. Some support for this hypothesis would be provided by the observation of significant differences between the vagotomized and sham-operated rats in the first replication, unlike the second replication.

EXPERIMENT 2

The results of the preceding experiment suggest that intragastric $CuSO_4$ is more likely to produce a reliable CTA in vagotomized animals than in sham-operated controls. One possible hypothesis to account for these results is to suggest that the effect of the vagotomy-induced delay in gastric emptying is to keep the $CuSO_4$ in the stomach for a longer period of time, thereby allowing it to cause the release of a toxin into the blood stream from which it can produce changes in the activity of the AP, the chemoreceptive trigger zone for emesis (Borison, 1974). Since $CuSO_4$ is not readily absorbed from the stomach, it may be that in the intact rat it passes too rapidly through the gastrointestinal system to be absorbed and, therefore, it is excreted before it can cause a CTA. In the vagotomized animal, to the contrary, it is held within the stomach for a long enough period to exert its toxic effects. One implication of this hypothesis is that the mechanism by which intragastric $CuSO_4$ would lead to the acquisition of a CTA would involve a humoral mechanism similar to that involved in the acquisition of a CTA produced by systemic injection of LiCl or other toxins (Rabin et al., 1983a; Ritter, McGlone & Kelly, 1980). If this hypothesis is correct, then it should be possible to disrupt a CTA produced by intragastric $CuSO_4$ in the vagotomized rat by lesions of the AP just as AP lesions disrupt the acquisition of taste aversions produced by exposure to ionizing radiation or by systemic injection of LiCl and other toxins. This experiment was designed to test the hypothesis of humoral mediation of the CTA produced by intragastric $CuSO_4$ in the vagotomized rat by studying the effects of AP lesions on the acquisition of a CTA in vagotomized rats.

Methods. The subjects were 45 naive male albino rats weighing 275–350 g at the time of initial surgery. They were divided into five groups: (1) AP lesion and vagotomy (AP + VGX, $n = 9$); (2) AP lesion and sham vagotomy (AP + sham, $n = 9$); (3) sham AP lesion and vagotomy (sham + VGX, $n = 7$); sham AP lesion and sham vagotomy (sham + sham, $n = 10$); sham lesion and sham vagotomy/saline intubation (sham + sham/saline, $n = 10$). The first four groups received intragastric intubations of $CuSO_4$ (5 mg/kg $\times$ $5H_2O$) with an infant feeding tube on the conditioning days, Days 1, 4 and 7, following ingestion of the sucrose solution, while the last group received intubation of an equivalent volume of isotonic saline on those days. Days 10 and 14 were test days and the sucrose was not followed by intubations of the unconditioned stimuli. A repeated treatment, single-bottle design was used to make this experiment equivalent to the preceding one. The AP and sham lesions were accomplished first. The rats were then given 2–3 weeks to recover before the vagotomy and sham vagotomy procedures were begun. Deprivation training was begun 4–6 weeks after completing the vagotomies. Verification

of the AP lesions was accomplished by examining thionin-stained sections taken through the brainstem and the vagotomies were verified by comparing the stomach weight/body weight ratios of the vagotomized rats with the appropriate controls.

Results and Discussion. The results are summarized in Fig. 3, which indicates that the only group that showed continuously decreased sucrose intake was the group of rats given the sham AP lesions combined with vagotomy. A two-way repeated measures analysis of variance showed that the main effect for surgery was highly significant, $F(4, 40) = 8.74$, $p < .001$. Because neither the main effect for the comparison for days across Days 4–14, $F(3, 120) = 0.27$, $p > .10$, nor the Surgery × Day interaction, $F(12, 120) = 0.94$, $p > .10$, was significant, the data for each group were collapsed across days for a more detailed analysis using orthogonal comparisons (Keppel, 1973). These comparisons showed that the sham + sham group intubated with $CuSO_4$ did not differ significantly from the sham + sham group intubated with saline, $F(1, 40) = 0.02$, $p > .10$, confirming the results of the previous experiments that intubation of $CuSO_4$ in intact rats does not reliably produce a CTA. The only comparisons that were significant were the comparisons between the sham + VGX and the other groups in the experiment, $F(1, 40) = 143.94$, $p < .001$, and between the sham + VGX and the AP + VGX groups, $F(1, 40) = 7.22$, $p < .05$). None of the other comparisons were significant.

As in the previous experiment, a CTA was observed only in the vagotomized animals with an intact AP, while lesions of the AP, which by themselves had no effect on sucrose intake, completely blocked the acquisition of a CTA in the vagotomized subjects. These results are, therefore, consistent with the hypothesis that the delay in gastric emptying

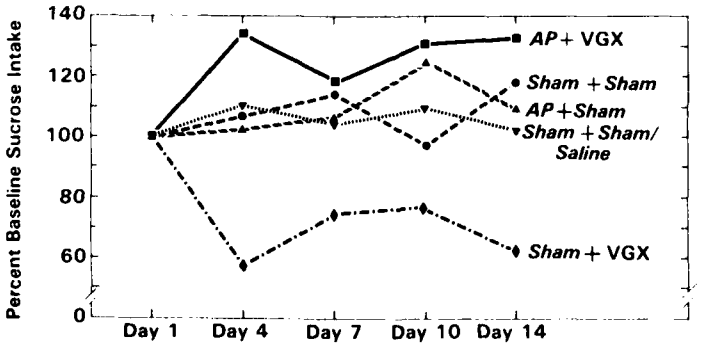

FIG. 3. Effects of repeated intragastric intubation of $CuSO_4$ (5 mg/kg × $5H_2O$) on the acquisition of a CTA in rats with lesions of the area postrema and/or vagotomy using a single-bottle design. The $CuSO_4$ was administered immediately following ingestion of a 10% sucrose solution on Days 1, 4, and 7. The saline group was administered intragastric saline instead of the $CuSO_4$ given all other groups.

produced by the vagotomy may permit the absorption of a toxin to which the AP is sensitive and which, in turn, mediates the acquisition of a CTA. It is not, however, certain what this toxin might be because preliminary analyses have indicated that the intubation of $CuSO_4$ in vagotomized animals, unlike intubation of LiCl, does not produce measurable increases in the serum levels of cupric ion.

The present results, which indicate that AP lesions by themselves have no effect on the acquisition of a CTA produced by intragastric $CuSO_4$ in intact animals, are in partial agreement with results reported by Coil and Norgren (1981). However, they reported that AP lesions failed to reverse a CTA produced by intragastric $CuSO_4$ in intact rats, whereas we have been unable to obtain a CTA under these conditions. Nonetheless, their finding that lesions of the AP disrupt the acquisition of a CTA produced by intravenous $CuSO_4$ would be consistent with the observation that these lesions disrupt the acquisition of a CTA produced by intraperitoneal $CuSO_4$ (Rabin, Hunt, & Lee, unpublished observations), as well as with the present results which show that AP lesions disrupt the CTA produced by intragastric $CuSO_4$ in vagotomized animals. It therefore seems that, under all conditions for which $CuSO_4$ may be used as an effective unconditioned stimulus for CTA learning, at doses of 5 mg/kg or less, either intravenous or intraperitoneal injections in intact rats or intragastric intubation in rats with subdiaphragmatic vagotomy, a similar AP-mediated mechanism serves as the underlying basis for the acquisition of a CTA.

GENERAL DISCUSSION

The present results are in conflict with those of Coil and her co-workers (Coil & Norgren, 1981; Coil et al., 1978) who reported that intragastric $CuSO_4$ produces a CTA that can be disrupted by cutting the vagus. In the present experiments it was not possible to reliably produce a CTA with intragastric $CuSO_4$ in intact rats, although a $CuSO_4$-induced CTA was reliably observed in rats with subdiaphragmatic vagotomy following intragastric administration. While several of the present experiments used procedures similar to those of Coil et al. (1978), they were not identical. It is not, however, likely that these procedural differences were responsible for the behavioral differences because the CTA is a robust effect that has not, in previous research, been affected by these variations in procedure. In addition, the observation that it was possible to produce taste aversions in rats with subdiaphragmatic vagotomies following treatment with intragastric $CuSO_4$, as well in intact rats given intraperitoneal $CuSO_4$ (Rabin et al., 1983b), would indicate that the procedures utilized in the present experiments were sufficient to produce a CTA under the right experimental conditions. As such, it is not obvious from the published literature what factors might have contributed to the different results.

The observation that lesions of the AP disrupt the acquisition of a CTA produced by systemic treatment with $CuSO_4$ in intact rats (Coil & Norgren, 1981; Rabin, Hunt, & Lee, unpublished observations) as well as by intragastric administration in vagotomized rats would be consistent with the hypothesis that a common mechanism underlies CTA learning produced by treatment with this dose of $CuSO_4$. Since the AP functions as the chemoreceptive trigger zone for emesis (Borison, 1974), the implication is that some common humoral factor underlies the acquisition of a $CuSO_4$-induced CTA, although the nature of that factor remains to be identified. Within this context, then, the role of vagotomy in the CTA produced by intragastric $CuSO_4$ would be to retain the $CuSO_4$ in the stomach for a period of time sufficient to allow for a $CuSO_4$-released toxin to get into the blood stream from which it can affect AP activity.

The observation in the present experiments that intragastrically administered $CuSO_4$ does not, by itself, produce a CTA in intact rats raises the question of the role of stomach irritation in CTA learning as well as the relationship between CTA and emesis. Because the functional effects of a CTA and emesis are similar, to limit the intake and/or absorption of toxic substances, it has been suggested that the mechanisms underlying both responses are similar (e.g., Rabin, Hunt & Lee, 1984). However, the observation that $CuSO_4$ produces a vagally mediated emetic response in dogs (Wang & Borison, 1951), but does not lead to the acquisition of a CTA in intact rats, would indicate that there are some differences between emesis and CTA learning in response to $CuSO_4$-induced gastric irritation. The present data do not allow a determination of whether this difference is due to species differences between dogs which are capable of emesis as opposed to rats which are not, or whether the difference is related to the nature of the response, emesis, which is an unconditioned response to treatment with a toxic unconditioned stimulus, as opposed to a CTA, which is a conditioned response to a conditioned stimulus. The present results might suggest that the acquisition of a CTA, in contrast to the emetic response, induced by gastric irritation requires a longer period of action by the toxin, conditions that are met by using vagotomized subjects.

In summary, the present experiments suggest that intragastric $CuSO_4$ is not a reliable unconditioned stimulus for the acquisition of a CTA in intact rats. However, when vagotomized rats were used a CTA was more consistently observed following intragastric administration of $CuSO_4$ at a dose of 5 mg/kg. The observation that the CTA induced by $CuSO_4$ in vagotomized rats could be disrupted by AP lesions suggests that the mechanism by which intragastric $CuSO_4$ can lead to CTA learning involves some humoral toxin, in a manner similar to CTA learning produced by LiCl or radiation, or by systemic treatment with $CuSO_4$. In this context, then, the role of the vagotomy is not to disrupt CTA learning, as suggested

by Coil et al. (1978), but rather to prolong the action of $CuSO_4$ in the gastrointestinal system and, thereby, produce the toxic conditions necessary for the acquisition of a CTA.

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Experimental Cell Biology
Editors: J.R. Rüttner, Zürich; G.V. Sherbet, Newcastle upon Tyne;
H. Ramseier, Zürich; L. Helson, New York, N.Y.; A. Wolsky, New York, N.Y.

Reprint
Publisher: S. Karger AG, Basel
Printed in Switzerland

Expl Cell Biol. *53*: 252–259 (1985)

Modulations in Mouse Hemopoiesis after Engraftment with Lewis Lung (3LL) Carcinoma Cells[1]

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Key Words. Lewis lung carcinoma (3LL) · Burst forming unit-erythroid · Colony forming unit-erythroid · Splenomegaly · Hematocrit · Leukocytosis

Abstract. Hemopoietic changes in male C57BL/6Cum BR mice engrafted with Lewis lung carcinoma (3LL) were evaluated between day 7, when palpable tumors were present, to day 30 postengraftment. All experimental animals demonstrated decreasing hematocrits (down 40% by day 30) with concurrent leukocytosis which by day 30 postengraftment had reached levels 13.4 times normal. The myelocytic/erythrocytic ratio for normal animals was 1:3 (bone marrow: spleen). The ratio for engrafted animals ranged between 10:1 and 40:1. This apparent shift in production priorities is even more significant in light of the fact that femoral bone marrow cellularity had decreased by 33% on day 17. Splenomegaly, evident by day 7, was seven times control by day 17. Clonogenic analysis of erythroprogenitor cell concentrations revealed an inverse relationship between bone marrow and spleen. 27 days after engraftment, splenic populations demonstrated significant increases in colony forming unit-erythroid (115-fold), burst forming unit-erythroid (7.4-fold), whereas bone marrow concentrations had decreased (6-fold). This report suggests that initiation of 3LL tumor in mice results in a change in the degree of hematopoietic priorities and participation of erythroid organs.

[1] Supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency under research work unit MJ 00030. The views presented in this paper are those of the authors. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

Research was conducted according to the principles enunciated in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animals Resources, National Research Council.

Introduction

Experimental animal models with transplantable nonmedullary solid tumors such as Ehrlich carcinoma [1, 2], adenocarcinoma [3, 4], and Lewis lung carcinoma [5–8] have shown dramatic perturbations in both marrow and spleen hemopoietic multipotential stem cells and committed progenitor cell populations. This report suggests that over a period of 7–30 days postengraftment a number of hematopoietic changes take place. Peripheral blood changes include alterations in cellularity, ratio, morphology, and significant changes in progenitor cell concentrations.

Materials and Methods

Mice

Male C57BL/6Cum BR mice, 12–16 weeks old (Cumberland View Farms, Clinton, Tenn.), free of lesions of murine pneumonia complex and of oropharyngeal *Pseudomonas* spp., were maintained on a diet of Wayne Lab-Blox food pellets and chlorinated water (11 ppm) available ad libitum. Mice were housed in filter-covered cages in a room with a 12-hour light-dark cycle.

Tumor

Lewis lung (3LL) carcinoma is a transplantable metastatic tumor that arose spontaneously in pulmonary tissues of a C57BL/6 mouse received in its 86th passage. The tumor line has been passaged in our laboratory since 1974. Tumor cells derived from 16-day-old tumors were routinely prepared for passage (as previously outlined in detail [6]) in 0.2 ml of 10% (v/v) concentration of cells in RPMI-1640 medium, injected subcutaneously in the left flank.

Experimental Design

Peripheral blood was obtained from anesthetized mice via cardiac puncture. Blood smears were stained with benzidine and counterstained with Wright's-Giemsa blood stains. The microplasma clot culture system, previously described in detail [7], was used to assess erythroid progenitor cells. Positive engraftments were palpable by day 7 and for that reason, day 7 was selected as the first examination day. Three independent studies were done, with 3 mice used per data point.

Results

Peripheral Blood

The red blood cell (RBC), hematocrit (Hct), and white blood cell (WBC) values of mice engrafted with tumor cells are shown in figure 1. Particularly evident are the conditions of anemia and leukocytosis. A decrease is noted in (fig. 1) packed cell volume (16%) by day 13 and a 41% decrease by day 30. Figure 2 demonstrates the pronounced leukocytosis (13.4 × normal, day 30) that occurs after engraftment.

Bone Marrow

By day 7 engraftment had caused bone marrow (BM) cellularity to decrease. Over the entire experimental time frame, cellularity decreased to 67% of normal. The data presented in figure 4 and table I suggest that a wave of nucleated erythroid cells is first observed between days 7 and 17, when granulocytic cellular elements were essentially within normal levels. This was followed by a decrease in nucleated erythroid cells and a concomitant increase in proliferative granulocytic cells (myeloblasts, promyelocytes, and myelocytes) between days 19 and 23. This rebound of activity in proliferative granulocyte cell activity was followed by an increase in nonproliferative granulocytic cells (metamyelocytes and mature granulocytes) between days 23 and 30.

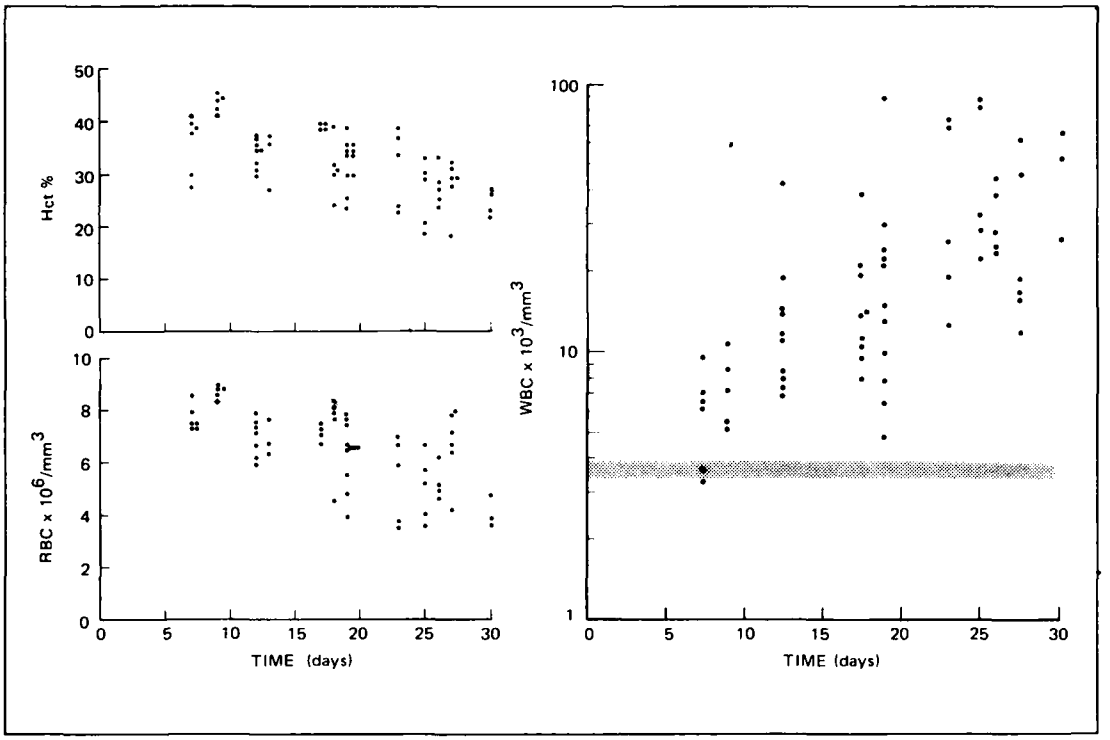

Fig. 1. Peripheral blood hemogram of male C57BL/6Cum mice engrafted with Lewis lung carcinoma cells. Each point represents a value for 1 mouse: Hct percentages, RBC counts $\times 10^6/mm^3$, and WBC counts $\times 10^3/mm^3$. Shaded area represents mean ± SEM values for normal mice (n = 9; Hct = 41.61 ± 0.55%; RBC = 8.27 ± 0.17 $\times 10^6/mm^3$; WBC = 3.67 ± 0.29 $\times 10^3/mm^3$).

Table I. M/E ratio of recognizable hemopoietic cells for femoral BM and spleen

Days after engraftment with 3LL tumor cells	BM	Spleen
9	6.2	0.24
12	8.9	0.33
18	3.75	0.38
23	10.0	–
25	38.5	–
27	6.4	0.16
30	3.7	–
Control (n = 9)	2.76	8.33

Table II. BFU-E/10^5 nucleated BM or spleen cells (SPL) plated at days 7 and 27 after engraftment of Lewis lung carcinoma cells (mean ± SEM values)

Experiment No.	Day 7[1]		Day 27[1]	
	BM	SPL	BM	SPL
1	08.0 ± 2.3	1.6 ± 0.5	10.6 ± 0.6	2.6 ± 1.2
2	24.6 ± 1.7	7.1 ± 1.1	11.0 ± 2.4	5.6 ± 0.3
3	40.6 ± 6.4	0.9 ± 0.3	14.3 ± 1.8	3.0 ± 0.4

Control values: BM 19.7 ± 1.9; SPL 0.5 ± 0.1; n = 9.

[1] Days 7 and 27 were selected as peaks of BFU-E activity.

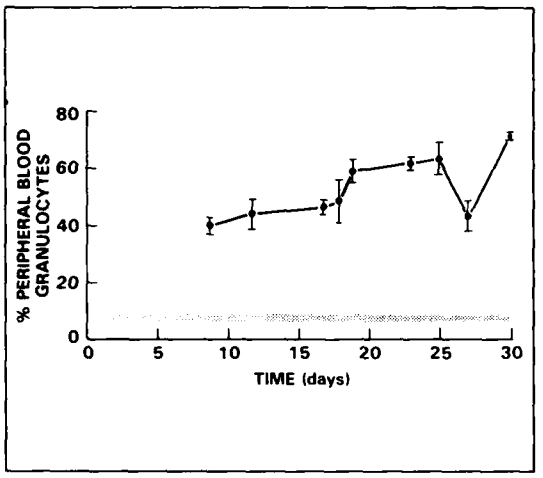

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Fig. 2. Peripheral blood percent granulocytes of male C57BL/6Cum engrafted with Lewis lung carcinoma cells. Values are presented as the mean ± SEM for three independent studies using at least 3 mice per time point. Shaded area represents mean ± SEM values for normal mice (n = 9; 8.0 ± 2.0%).

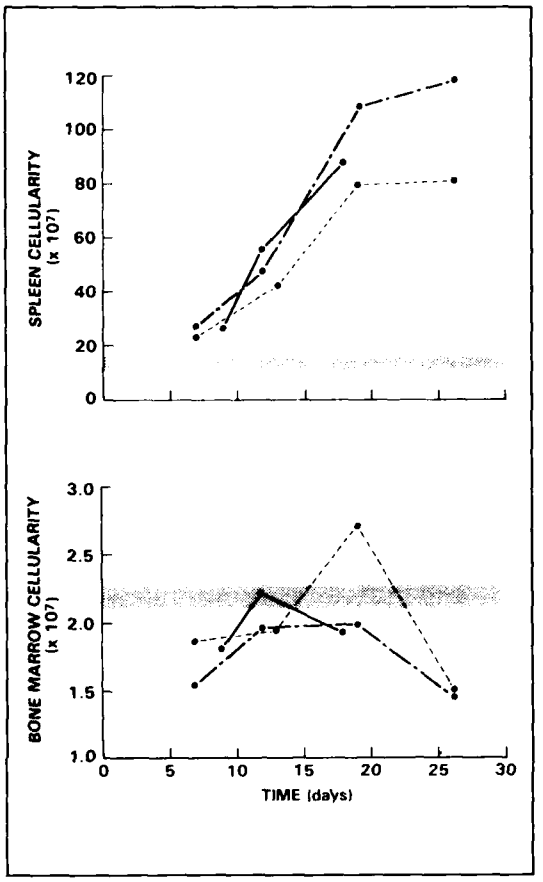

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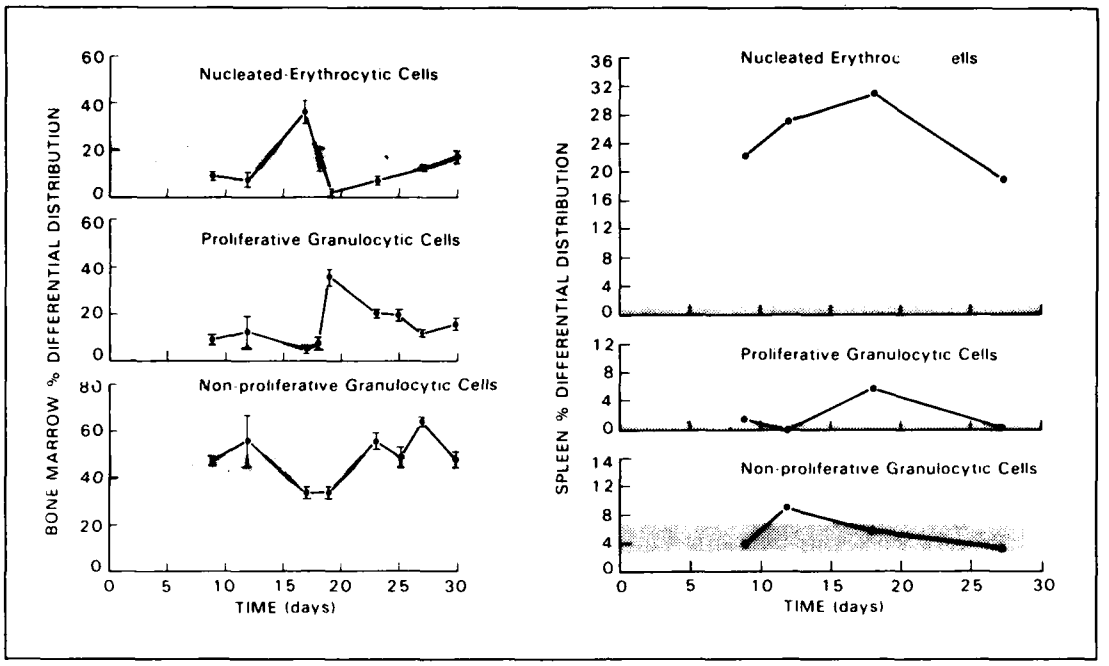
Fig. 3. BM and spleen cellularity of male C57BL/6Cum mice engrafted with Lewis lung carcinoma cells. Each line represents an independent study using at least 3 mice per time point studied. Shaded areas indicate mean ± SEM values for normal mice (n = 9; BM = 2.18 ± 0.09 × 10^7 nucleated cells and spleen = 13.93 ± 1.58 × 10^7 nucleated cells).

Data from clonogenic assays support the microscopic observations of hemopoietic cellular redistribution of the femoral marrow after engraftment with tumor cells. Numbers of erythroid progenitor cells, burst forming unit-erythroid (BFU-E) on day 7 were almost 2-fold greater than values obtained at day 27 (table II). High numbers of BFU-E (day 7) preceded the wave of activity of the more mature erythroid progenitor cells, colony forming unit-erythroid (CFU-E), observed between day 7 and day 19 (fig. 5). By day 27, a 6-fold decrease was seen in the numbers of CFU-E/10^5 marrow cells compared to control mice. Suppression of BM erythroid elements in the engrafted mouse is further supported by the results in one of the three replicate studies, in which both erythroid progenitor cells (BFU-E, table II and CFU-E, fig. 5) were already significantly suppressed by day 7 to below normal values.

Spleen

Splenic cellularity (day 7) had increased by 50%, and was 6.9-fold greater by day 17 (fig. 3). Unlike the distribution of the marrow



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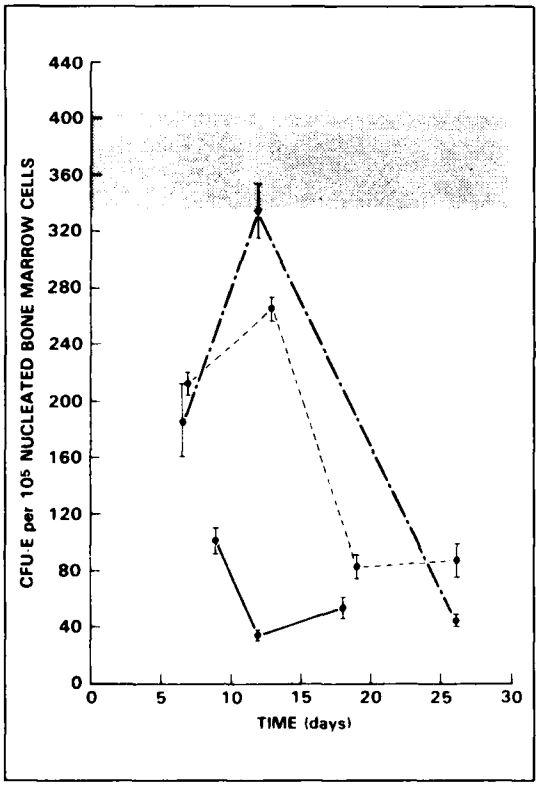

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Fig. 4. BM and spleen percent differential distribution of hemopoietic cell types. Proliferative granulocytic cells include myeloblasts, promyelocytes, and myelocytes. Nonproliferative granulocytic cells include metamyelocytes and mature granulocytes. Values are presented as the mean ± SEM for BM and mean for spleen of three replicate studies of male C57BL/6Cum mice engrafted with Lewis lung carcinoma cells using at least 3 mice per time point for each study. Shaded areas are mean ± SEM values for normal mice (n = 9). BM: proliferative = 7.3 ± 1.3%; nonproliferative = 44.0 ± 6.0%; nucleated erythroid = 18.6 ± 6.2%. Spleen: proliferative = 0.5 ± 0.5%; nonproliferative = 5.0 ± 2.3%; nucleated erythroid = 0.6 ± 0.6%).

Fig. 5. CFU-E values/10^5 BM cells from male C57BL/6Cum mice engrafted with Lewis lung carcinoma cells for each of three replicate studies using at least three mice for each time point per study. Values are presented as the mean ± SEM. Shaded areas are mean ± SEM values for normal mice (n = 9; BM = 360 ± 93/10^5 cells).

Table III. Overview of hemocytopoietic changes in the peripheral blood, femoral marrow, and spleen in mice after engraftment with Lewis lung carcinoma cells

Time after engraftment with tumor cells	
Day 7	Day 30
Peripheral blood	
19% decrease in Hct	41% decrease in Hct
16% decrease in RBC	50% decrease in RBC
4.5-fold increase in WBC	13.4-fold increase in WBC
5-fold increase in % granulocytes	7.5-fold increase in % granulocytes
Spleen	
6.6-fold increase in cellularity	6.9-fold increase in cellularity
Decrease in M/E ratio	decrease in M/E ratio
Increase in nonproliferative granulocyte cells	increase in proliferative granulocyte cells
Increase in nucleated erythroid cells	increase in nucleated erythroid cells
115-fold increase in CFU-E	7.4-fold increase in BFU-E
Increase in CFU-S[1]	4.7-fold increase in MEG-CFC
Increase in GM-CFC[1]	
Increase in M-CFC[1]	
Bone Marrow	
8.7% decrease in cellularity	33% decrease in cellularity
Increase in M/E ratio	increase in M/E ratio
Increase in nonproliferative granulocyte cells	increase in proliferative granulocyte cells
Increase in nucleated erythroid cells	6-fold decrease in CFU-E
72% decrease in BFU-E	no change in MEG-CFC
Increase in GM-CFC[1]	
Increase in M-CFC[1]	

[1] *Ledney* et al. [1981].

hemopoietic cells, the splenic myelocytic/erythrocytic (M/E) ratio was less than 0.38 throughout the study, compared to a control M/E ratio of 8.33 (table I). An increase of splenic granulocytic activity occurred between day 7 and day 27, with an increase in nonproliferative granulocytic cells between days 7 and 17, followed by a more pronounced increase in proliferative granulocytic cells between days 12 and 27 (fig. 4).

Data from in vitro culture assays supports the microscopic data of splenic hemopoietic cellularity. The concentration of BFU-E/10^5 spleen cells plated appeared to reach two significant peaks of activity, the first at day 7 and the second at day 27 (table II). Figure 6 presents the data for spleen CFU-E. 12 days after engraftment, the concentration of CFU-E/10^5 spleen cells was 30–40 times greater than values for control mice.

Discussion

This paper reports modulations in murine hemopoiesis after engraftment with Lewis lung carcinoma cells. Throughout the study, there appears to be a causal relationship between tumor growth and the following: splenomegaly, medullary hypocellularity, peripheral blood neutrophilia and erythroid anemia, increased splenic erythrocytopoiesis and increased marrow granulocytopoiesis (table III). Portions of these observations are consistent with previously reported results. Peripheral blood neutrophilia refelected enhanced granulocytic activity of the bone marrow and spleen, suggested by the high numbers of marrow- and spleen-derived GM-CFU and the percent early recognizable granulocytic cellular elements. The occurrence of identifiable early granulocytic cells in a biphasic manner suggested the existence of a high concentration of granulocyte-stimulating factors that induce granulocyte differentiation. When the percentage of nonproliferative cells began to descend, a simultaneous increase occurred in the proliferating granulocytic cell compartment. The increase in GM-CFU observed on days 12–14 was more than likely responsible for the appearance of proliferating granulocytic cells observed during days 19–27 in the bone marrow and during days 12–27 in the spleen. The increase in marrow leukocytopoiesis was possibly at the expense of erythroid activity, indicated by an increase in marrow M/E ratio and a decrease in concentration of marrow-derived BFU-E and CFU-E.

The peripherally demonstrated anemia may be due to a loss, or change, in RBC production, an increase in the destruction of RBC, or changes in the plasma volume. Our data suggest that the peripheral anemia may be due in part to changes in site(s) of RBC production. Data reported here indicate a pronounced compensatory increase in splenic erythrocytopoiesis. However, this change of production, both alone and in combination with other factors, was unable to prevent anemia.

Our data are consistent in part with a number of publications that report hematopoietic effects of tumors and/or their factors on granulocytopoiesis [1, 3, 8–14] and imply that 3LL may induce release of substances which regulate the blood cell-forming tissues or the inductive microenvironmental factors.

Acknowledgment

The authors wish to thank Ms. *Junith Van Deusen* for her editorial assistance in the preparation of this manuscript.

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Received: September 6, 1984
Accepted: October 5, 1984

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